Familiar mutualist interactions during biological invasions: Consequences for invaders and impacts on natives

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DECLARATION

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Thesis Summary

Mutualisms are important for plant survival but are easily disrupted when plants are introduced into new environments. This acts as a strong barrier to establishment success. However, non-native plants can form novel mutualisms with resident species or, when co-introduced, can maintain familiar associations. Plants that co-invade ecosystems with their mutualists usually impact native species more severely than invasive plants that form novel associations. Invasive Australian acacias (genus *Acacia* Mill.) make use of both mutualist reassembly pathways to facilitate their invasion success in nutrient poor environments. These acacias frequently alter (a)biotic soil conditions, e.g. via soil nutrient enrichment, leading to positive feedbacks.

The first aim of this thesis was to determine the relative contributions of novel vs familiar rhizobial associations to the establishment success of *Acacia saligna* across different soils in South Africa's Core Cape Subregion. As a second aim, I also investigated whether leaf litter of *Acacia saligna* benefits its seedlings' establishment under competition with a native legume, and how this may act synergistically with familiar rhizobial associations to improve the competitive ability of the species.

For the first aim, I grew *A. saligna* and the native legume, *Psoralea pinnata*, in a glasshouse experiment in five different CCR soils under two inoculum addition treatments. Australian bradyrhizobia isolated from acacias were used as inocula. Various performance measures were recorded and next-generation sequencing (NGS) barcoding methods used to identify rhizobia associating with the two legumes across treatments. For both legumes, few significant inoculum effects were found for any performance measures. Plant performance responded more strongly to soil type. Barcoding revealed that *A. saligna* and *P. pinnata* were predominantly associating with Australian *Bradyrhizobium* and native *Mesorhizobium*, respectively, irrespective of treatment x soil combination.

For the second aim, I grew *A. saligna* and *P. pinnata* together in pots containing *Psoralea*-conditioned soils and exposed them to Australian inoculum and acacia topsoil (which represented acacia leaf litter) treatments in a fully factorial design. I incorporated data for seedlings grown in the same soil from the glasshouse experiment discussed under aim one to compare performances when grown alone vs in mixture so as to determine how Australian bradyrhizobia may facilitate acacia performance. I also compared the performances of each

legume grown together in mixture between the four inoculum and topsoil treatment combinations. Overall, I found no significant inoculum or topsoil effects on the performance of either legume. NGS revealed similar rhizobial associations as in the first experiment.

Overall, this thesis revealed that both legume species formed familiar associations regardless of *Acacia-Bradyrhizobium* cointroductions or acacia-mediated positive feedbacks. This suggests that *P. pinnata* may be valuable for restoration projects after acacia clearing. The presence of Australian bradyrhizobia in all soils (including uninoculated soils) also suggests that these strains are already present and proliferating within the CCR, and can thereby facilitate future Australian acacia invasions as mutualist absence may no longer be a barrier to acacia establishment success.

Tesis Opsomming

Mutualismes is belangrik vir plantoorlewing, maar word gewoonlik tydens plantvrystellings ontwrig en kan dus 'n hindernis wees vir die suksesvolle vestiging van uitheemse spesies. Uitheemse plante kan egter mutualiste verkry deur middel van nuwe assosiasies met inwonende mutualiste, of deur middel van bekende assosiasies (dit wil sê, mutualiste wat saam met plante vrygestel is). Altwee hierdie padweë vir die herontmoeting van uitheemse plante en hulle mutualiste het voordele vir indringerspesies en hul gepaardgaande impakte op inheemse spesies. Beide sal hoër wees tydens bekende assosiasies. Uitheemse Australiese akasias het hul mutualistiese rhizobië verkry deur middel van beide nuwe en bekende assosiasies, wat hul indringingsukses in voedingsstof-arm omgewings bevoordeel. Akasias verander ook (a)biotiese toestande tydens indringing wat lei tot positiewe terugvoermeganismes (bv. die verryking van grond voedingsstowwe deur middel van blaarvullis).

Die eerste doel van hierdie proefskrif was om die relatiewe bydraes van nuwe, teenoor bekende, rhizobiese assosiasies tot die vestigingsukses van *Acacia saligna* in verskillende grondtipes in die Kaapse Kern Subomgewing (KKS) van Suid-Afrika te bepaal. As 'n tweede doel het ek ook ondersoek ingestel om te bepaal of blaarvullis van *Acacia saligna* dié spesie se vestiging bevoordeel onder kompetisie met 'n inheemse peulplant, en hoe dit sinergisties kan werk met bekende rhizobiese assosiasies om die mededingingsvermoë van die spesie te verbeter.

Vir die eerste doel het ek *A. saligna* en die inheemse peulplant, *Psoralea pinnata*, gekweek in 'n kweekhuis-eksperiment in vyf verskillende KKS-gronde onder twee toevoegings van inenting. Australiese bradyrhizobië, geïsoleer vanuit akasias, is gebruik as entstof. Verskeie plantegroeimetings is aangeteken en volgende generasie basisvolgordebepaling (NGS) is gebruik om rhizobië te identifiseer wat met die twee peulplante geassosieer was. Vir beide peulplante is min beduidende effekte van entstowwe vir enige plantegroeimetings gevind. Plantprestasies het sterker gereageer op grondsoort. NGS het ook getoon dat *A. saligna* en *P. pinnata* hoofsaaklik assosieer met onderskeidelik Australiese *Bradyrhizobium* en inheemse *Mesorhizobium*, ongeag van entstof behandeling x grond kombinasie.

Vir die tweede doel het ek *A. saligna* en *P. pinnata* saam gegroei in potte wat *Psoralea*-gekondisioneerde grond bevat het en dié blootgestel aan Australiese entstof en akasie bogrond (verteenwoordigend van akasia-blaarvullis). Ek het plantegroeidata versamel vir saailinge wat

in dieselfde grond gekweek was in die eksperiment wat onder doel 1 bespreek was. Dit het my toegelaat om data te vergelyk tussen plante wat alleen en in 'n mengsel gegroei was om vas te stel hoe Australiese bradyrhizobië die groei en kompeterende vermoë van akasië beinvloed. Ek het ook die groei van beide peulplante vergelyk tussen die vier kombinasies vir inenting en bogrond onder kompetisie. Oor die algemeen het ek geen beduidende effekte van inenting of bogrond op die groei van beide die peulplante gevind nie. NGS het soortgelyke rhizobiese assosiasies aangetoon as wat ek in die eerste eksperiment bepaal het.

Oor die algemeen het hierdie tesis bevestig dat beide peulplantspesies bekende assosiasies met rhizobië gevorm het, ongeag van die teen woordigheid van Australiese *Acacia* en *Bradyrhizobium*, of hul positiewe terugvoermeganismes. Dit dui daarop dat *P. pinnata* waardevol kan wees in restourasieprojekte na die verwydering van akasia. Die aanwesigheid van Australiese bradyrhizobië in alle gronde (insluitend oningeënte gronde) dui ook daarop dat hierdie bakterieë reeds in die KKS voorkom, en sodoende toekomstige Australiese akasia vrylatings kan bevoordeel, aangesien die onderlinge afwesigheid van effektiewe mutualiste nie meer 'n hindernis is huk vestigingssukses nie.

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Chapter 1

General background

Pathways of mutualism reassembly in novel environments during biological invasion

What allows some plants to become successful invaders and others not remains a central question in invasion biology (Funk, Standish, Stock, *et al.*, 2016; Pyšek, Jarošík, Hulme, *et al.*, 2012; Rejmánek & Richardson, 1996). In many instances, the success of potential invaders is dependent upon the (a)biotic interactions they experience within the non-native range. Given their apparent role in the completion of many plants' life cycles, it is now widely accepted that the formation of successful mutualistic associations in novel environments, or lack thereof, may be a key determinant of the establishment success of introduced plants (Richardson, Allsopp, D'Antonio, *et al.*, 2000). These include mutualisms associated with pollination, seed dispersal, mycorrhization, etc. (Richardson, Allsopp, *et al.*, 2000). For example, many pine (family Pinaceae) introductions initially failed in Southern Hemisphere countries due to a lack of compatible ectomychorrizal fungal partners (Policelli, Bruns, Vilgalys, *et al.*, 2019). It was only after the introduction of these fungal mutualists that pines established and became widespread invaders in these countries (Richardson, Williams & Hobbs, 1994).

Like all interaction types, mutualisms span a continuum of specificity (Bascompte, 2009). At the two extremes we find associations that are highly specific and involve only two partners, or associations that are highly promiscuous, i.e. where a single plant associates with several symbionts or vice versa (Sprent, 2007). In between these two extremes lies an infinite number of outcomes (Sprent, 2007). The strength and specificity of mutualistic interactions are moulded by the degree of co-evolutionary history shared between the two interacting partners (Ehrlich & Raven, 1964). However, for introduced species, co-evolution is an inadequate explanation for the often rapid formation of mutualistic interactions in novel community contexts (Petanidou, Kallimanis, Tzanopoulos, *et al.*, 2008). A more likely explanation is based on Janzen's (1985) theory of ecological fitting, i.e. that hosts may switch their mutualistic partners in response to context-dependent changes, such as availability of effective mutualists. Intuitively, mutualistic promiscuity is advantageous for species introduced to novel ranges, as this increases their likelihood of forming effective associations with symbionts they have never

encountered before (Parker, 2001; Le Roux, Hui, Keet, *et al.*, 2017). On the other hand, it is possible that some non-native species are co-introduced with their mutualists into their new ranges (e.g. Crisóstomo, Rodríguez-Echeverría & Freitas, 2013; Prior, Robinson, Meadley Dunphy, *et al.*, 2014; Ramírez & Montero, 1988). This may be particularly advantageous for the successful establishment of non-native species with highly specific mutualist requirements (Le Roux *et al.*, 2017).

While promiscuity would be advantageous for non-native species introduced without their co-evolved mutualists, novel associations may nonetheless negatively impact their establishment success and subsequent invasion performance, especially if compatible mutualists occur in low abundances initially or have low effectiveness (Le Roux *et al.*, 2017). In such cases, the focal mutualists will need to be selected for, and amplified, by the introduced species (Heath & Tiffin, 2007). Additionally, co-occurring native plants may successfully compete with introduced species for available mutualists through superior (and potentially co-evolved) mutualist attraction – a form of biotic resistance (Le Roux *et al.*, 2017). Although these effects may diminish over time as non-native species' densities increase, the fine-tuning of novel associations may act to increase lag times (i.e. stage between establishment and invasion). Moreover, such prolonged lag phases, and possible ineffectiveness of novel associations, may translate into lower rates of accrual, and extent of, ecological impacts caused by the invasive species (Le Roux *et al.*, 2017). In contrast, when co-evolved plants and their mutualists are co-introduced, it is expected that the advantage of increased host promiscuity is less significant in facilitating establishment and spread of the introduced plant as their familiar associations are readily available. It has been suggested that plants that have been co-introduced with their mutualists can establish and spread more rapidly, and their ecological impacts may accrue faster and may be more severe, compared to those plants relying on novel associations (Le Roux *et al.*, 2017). Under both novel and familiar associations, positive-feedback loops may be generated which act to enhance the invader's performance, while simultaneously suppressing native competitors (Fig. 1.1). For example, changes in abundances of mutualists may be amplified by indirect invader-induced effects, e.g. changes in soil abiotic and biotic conditions due to increased leaf-litter input and subsequent nutrient enrichment (e.g. Yelenik, Stock & Richardson, 2004). As mentioned, under novel associations, initial ecological impacts may be less severe and will take longer to accrue as these novel mutualistic associations and/or adaptations may require fine-tuning over time. On the other hand, the

formation of familiar associations will be less limiting and will therefore have more profound ecological impacts. Additionally, these impacts are likely to accrue more rapidly as a result of positive feedbacks between densities of non-native mutualists and their co-evolved non-native host plants, in turn enhancing competition between native and non-native mutualists and plants. Overall, the net effects of these positive feedbacks act to enhance the impacts common under both mutualist association scenarios i.e. direct and indirect plant-plant effects, plant-mutualist effects, and disruption of native plant-mutualist interactions (Le Roux *et al.*, 2017).

Legume-rhizobia mutualisms during invasion

Evidence from invasive legumes and their mutualistic nitrogen-fixing bacteria, known as rhizobia, show that the formation of both novel and familiar rhizobial associations are common strategies for acquiring mutualists in novel environments. Research on the impact of legume-rhizobium mutualisms on non-native species establishment traditionally lagged behind that on other mutualistic interactions, but has gained momentum over the past two decades. Legumes (family Fabaceae) are the third largest family of flowering plants (Daehler, 1998), divided into three distinct sub-families: Caesalpinioideae, Mimosoideae and Papilionoideae (Sprent, 2007). Overall, legumes are widespread in that they occur on almost every continent and are also diverse in terms of growth forms, ranging from herbaceous to woody shrubs and trees (Daehler, 1998; Sprent, Ardley & James, 2017).

As invasives species, legumes from the Caesalpinoid and Mimosoid subfamilies are over-represented as invaders of natural areas (Daehler, 1998), with 121 woody legume taxa recognised as invasive (Richardson & Rejmánek, 2011). These often cause severe ecological impacts (e.g. Gaertner, Biggs, Te Beest, *et al.*, 2014; Medina-Villar, Rodríguez-Echeverría, Lorenzo, *et al.*, 2016), including changes to soil chemistry and nutrient composition and reducing native biodiversity (Le Maitre, Gaertner, Marchante, *et al.*, 2011; Yelenik *et al.*, 2004). Many functional traits have been linked to the invasiveness of legumes, such as their rapid growth rates, generalist insect pollination and ability to reproduce vegetatively (Hughes & Styles, 1989). Mutualistic associations with nitrogen-fixing bacteria is touted as paramount to the high invasion success of legumes (Daehler, 1998; Parker, Malek & Parker, 2006; Yelenik, Stock & Richardson, 2007).

Rhizobia are not monophyletic, falling in both the *alpha*- and *beta*-Proteobacteria classes (Sprent, 2007). The classical legume-associating rhizobia belong to five different genera within these two groups: *Bradyrhizobium*, *Ensifer* (formely *Sinorhizobium*), *Rhizobium* and *Mesorhizobium* of the *alpha*-Proteobacteria and *Paraburkholderia* (formely *Burkholderia*; Sawana, Adeolu & Gupta, 2014) of the *beta*-Proteobacteria (Bontemps, Elliott, Simon, *et al.*, 2010; Sawada, Kuykendall & Young, 2003; Weir, Turner, Silvester, *et al.*, 2004), although many more genera exist (see Peix, Ramírez-Bahena, Velázquez, *et al.* (2015) and Sprent *et al.* (2017) for review). Rhizobia are free-living soil bacteria capable of forming specialized structures, known as nodules, on the roots and, less frequently, the stems of most legumes. Biological nitrogen fixation (BNF) occurs within these nodules whereby rhizobia reduce inorganic atmospheric nitrogen into organic forms, such as ammonium, which is transferred to the legume host in exchange for carbon-rich photosynthates.

The specificity of legume-rhizobia interactions is driven by intricate molecular communication (Perret, Staehelin & Broughton, 2000), and thus the genotypes (Barrett, Bever, Bissett, *et al.*, 2015), of interacting partners. Generally, nodulation is initiated by the legume roots exuding (iso)flavonoids into the rhizosphere to stimulate the expression of rhizobial symbiotic genes, known as Nodulation (*nod*) genes, which are responsible for initiating the process of root nodule formation. *Nod* genes are located on mobile genetic elements, such as symbiotic plasmids or genetic islands (Rogel, Ormeño-Orrillo & Martinez Romero, 2011). Their expression is almost always regulated by *nodD* which acts as the sensor to a legume signal and is ubiquitous among rhizobia (Perret *et al.*, 2000). The activation and expression of the *nodD* gene leads to the cascading stimulation of the *nodABC* gene complex (Le Roux *et al.*, 2017). These genes are responsible for the production of nodulation enzymes as well as Nod factors, a family of lipo-chito-oligosaccharides (LCOs). These Nod factors are secreted by the rhizobia and stimulate the root hairs – unicellular extensions of the root epidermis – to curl, through the reorientation of their cell wall growth. Additionally, Nod factors also stimulate the formation of tubular structures within the curling root hair through which the rhizobia may enter, known as an infection thread, which leads to their entrapment, and ultimately to the formation of a nodule (Fig. 1.2) (Perret *et al.*, 2000; Le Roux *et al.*, 2017). Variation exists between different legume-rhizobium interactions in terms of the excreted plant compounds as well as *nod* genes and their strain-specific combinations (e.g. Lira (Jr.), Nascimento & Fracetto, 2015), all of which contribute to the specificity of the association.

Rhizobial mutualisms have often been suggested to be a limiting factor in the successful establishment of non-native legumes (Simonsen, Dinnage, Barrett, *et al.*, 2017), with legumes exhibiting high levels of specificity often being more limited in terms of spread than generalist legumes (Harrison, Simonsen, Stinchcombe, *et al.*, 2018; Klock, Barrett, Thrall, *et al.*, 2015). Unsuprisingly, non-native legumes forming novel associations also often display high levels of symbiotic promiscuity and they frequently associate with compositionally-different rhizobia compared to their native ranges (e.g. Australian *Acacia* spp., *Cytisus* spp., *Leucaena* spp. and *Robinia* spp. in Brazil – de Faria & de Lima, 1998; *Acacia pycnantha* in South Africa – Ndlovu, Richardson, Wilson, *et al.*, 2013; *Trifolium* spp. in New Zealand – Shelby, Duncan, van der Putten, *et al.*, 2016). On the other hand, many legumes associate with identical rhizobia in their native and non-native ranges (e.g. *Cytisus scoparius* in North America – Horn, Parker, Malek, *et al.*, 2014; Australian *Acacia* spp. – Warrington, Ellis, Novoa, *et al.*, 2019, indicative of cointroduction. The link between promiscuity, cointroduction, and invasiveness is elegantly illustrated by the globally invasive legume genus *Mimosa* (*M. pudica*, *M. pigra* and *M. diplotricha*), where independent cointroductions of these species with co-evolved native rhizobia have been documented in Australia (Parker, Wurtz & Paynter, 2007), China (Liu, Wei, Wang, *et al.*, 2012), India (Gehlot, Tak, Kaushik, *et al.*, 2013), and Taiwan (Chen, James, Chou, *et al.*, 2005). In India, invasive *M. pudica* are only able to nodulate with co-introduced rhizobia and appear unable to utilize the rhizobial strains of a co-occurring, and endemic, Indian *Mimosa* species (Gehlot *et al.*, 2013; Melkonian, Moulin, Béna, *et al.*, 2014). This highlights the importance of cointroductions of familiar rhizobia in the invasion success of legumes with highly specific legume-rhizobium requirements.

While the genotypes of the interacting partners determines the specificity of legume-rhizobia interactions, these interactions can range from beneficial to neutral and even to suboptimal (Bronstein, 2009) which, in turn, is dependent on a variety of factors. For example, the benefits legumes receive from rhizobia are dependent on soil nitrogen levels, and whether these meet their nutrient demands (e.g. Barrett, Broadhurst & Thrall, 2012). Therefore, the importance of rhizobial mutualists in facilitating non-native species establishment and subsequent impacts on native species, are expected to be more intense in low nutrient environments (Keller & Lau, 2018; Lau, Bowling, Gentry, *et al.*, 2012). Rhizobia can vary from being mutualistic (when they benefit host legumes) to parasitic (when they colonize

legume root nodules without effectively providing fixed nitrogen to their host, i.e. cheater strains) (Denison & Kiers, 2004). Under high nutrient conditions, it is energetically more costly for legumes to acquire nitrogen via BNF compared to directly from the soil (Graham, 1992), providing oppurtunities for cheater strains to colonise legumes. However, many legumes have the ability to sanction rhizobial associations by limiting the supply of oxygen and photosynthates to ineffective nodules (Kiers, Rousseau, West, *et al.*, 2003). Sanctioning also allows legumes to select the most effective strains in low nutrient soils that harbour a diversity of rhizobia (Bever, 2015; Denison, 2000; Kiers *et al.*, 2003). Therefore, the benefits derived from rhizobial associations are largely context-dependent and can be driven by a variety of (a)biotic conditions (Bever, 2015; Lau *et al.*, 2012; Parker, 2001).

Australian *Acacias* in the Core Cape Subregion (CCR) of South Africa

Australian acacias in the genus *Acacia* Mill. *sensu stricto* (Leguminosae subfamily, Mimosoideae, formerly *Acacia* subgenus Phyllodineae DC; Maslin, 2008) have been widely studied, both for their economic value in agroforestry sectors as well as their invasion success and severe ecological impacts globally (Richardson, Carruthers, Hui, *et al.*, 2011). Currently, 23 *Acacia* spp. are recognised as invasive worldwide, with most of these found in semi-arid and nutrient-poor Mediterranean-type ecosystems, such as South Africa's Core Cape Subregion (CCR) (Le Maitre *et al.*, 2011; Richardson & Rejmánek, 2011).

Acacias have been described as ‘transformer’ species due to their ability to substantially change the character, structure and functioning of the ecosystems they invade and becoming active agents in ecosystem-forming processes through, for example, altered fire regimes (Richardson *et al.*, 2000; Marchante *et al.*, 2015). Different functional traits of acacias act in synergy to generate positive-feedbacks that, in turn, aid their invasion success through increasing their competitive ability and impact accrual on native species (Le Maitre *et al.*, 2011; Morris, Esler, Barger, *et al.*, 2011). Of these impacts, the most severe include changes in above- and belowground communities through the formation of monospecific stands, increased leaf-litter input, altered microclimates through increased shading, changes in soil moisture regimes and, lastly, changes in soil nutrient contents (Gaertner, Den Breeyen, Hui, *et al.*, 2009; Mostert, Gaertner, Holmes, *et al.*, 2017; Yelenik *et al.*, 2004). The majority of these impacts are attributed to a few key traits, including rapid growth rates and leaf-litter production, the capacity to accumulate a large amount of biomass, and the production of large and persistent

seed banks (Yelenik *et al.*, 2007). These functional traits, and the resultant ecological impacts they cause, are intrinsically linked to the ability of acacias to efficiently acquire nutrients from even the most nutrient poor environments (Young & Young, 2001). This is often attributed to their ability to fix atmospheric nitrogen and therefore, by default, their association with mutualistic rhizobia (Daehler, 1998; Parker *et al.*, 2006; Yelenik *et al.*, 2007)

Characteristically, Australian acacias are predominantly nodulated by members of the slow-growing genus *Bradyrhizobium* (Marsudi, Glenn & Dilworth, 1999; Rodríguez-Echeverría, Le Roux, Crisóstomo, *et al.*, 2011). However, they have also been found to form effective associations with fast growing strains, e.g. *Rhizobium* (Rodríguez-Echeverría *et al.*, 2011) and *Mesorhizobium* (Crisóstomo *et al.*, 2013) and *Paraburkholderia* (Ndlovu *et al.*, 2013). Additionally, although acacias are seemingly promiscuous hosts (Andrews & Andrews, 2017; Keet, Ellis, Hui, *et al.*, 2017; Ndlovu *et al.*, 2013; Rodríguez-Echeverría *et al.*, 2011), differences in legume-rhizobium mutualist specificity have been identified between different *Acacia* species (e.g. Birnbaum, Barrett, Thrall, *et al.*, 2012; Burdon, Gibson, Searle, *et al.*, 1999; Hoque, Broadhurst & Thrall, 2011; Thrall, Slattery, Broadhurst, *et al.*, 2007). There is some evidence to suggest that acacias form novel rhizobial associations in some of their non-native ranges (e.g. Birnbaum *et al.*, 2012; Klock, Barrett, Thrall, *et al.*, 2016; Ndlovu *et al.*, 2013). However, invasiveness of the group does not appear to be linked with symbiotic promiscuity or effectiveness (Keet *et al.*, 2017). Rather, their nodulation success appears to be predominantly attributed to the high levels of cointroduction with their co-evolved rhizobia into novel environments (e.g. Portugal – Crisóstomo *et al.*, 2013; Rodríguez-Echeverría, 2010; South Africa – Ndlovu *et al.*, 2013; Le Roux, Mavengere & Ellis, 2016; Warrington *et al.*, 2019; New Zealand – Weir *et al.*, 2004). In the above examples, co-introduced bradyrhizobial strains are phylogenetically distinct from rhizobia isolated from co-occurring native legumes. The high incidence of cointroduction of acacias and their rhizobia is perhaps unsurprising given that many acacias have been imported into various countries, particularly the Western Cape of South Africa, and New Zealand, for various ornamental and agroforestry purposes (Richardson *et al.*, 2011). Consequently, rhizobia may have been accidently introduced along with imported seeds/seedlings, or purposefully to promote the establishment and growth of the seedlings (Marques, Pagano & Scotti, 2001).

Acacia invasions in South Africa's CCR represent an interesting case to study the effects of novel vs familiar rhizobial associations on non-native legume performance and their impacts on native biodiversity. All five of the classical legume-nodulating rhizobial genera, i.e. *Paraburkholderia*, *Mesorhizobium*, *Rhizobium*, *Ensifer* and *Bradyrhizobium*, have been found within the CCR and in association with native CCR legumes (Beukes, Venter, Law, *et al.*, 2013; Elliott, Chen, Bontemps, *et al.*, 2007; Gerding, O'Hara, Bräu, *et al.*, 2012; Hassen, Bopape, Habig, *et al.*, 2012; Kanu & Dakora, 2012; Kock, 2004; Lemaire, Dlodlo, Chimphango, *et al.*, 2015; du Preez, 2019). However, there are differences in the predominance of strains associating with native CCR legume genera. For example, *Bradyrhizobium* (the preferred symbionts of acacias), *Rhizobium* and *Ensifer* strains are generally found in low abundances in the CCR (Lemaire *et al.*, 2015), while *Paraburkholderia* and *Mesorhizobium* are the predominant genera associated with CCR legumes (Beukes *et al.*, 2013; Gerding *et al.*, 2012; Lemaire *et al.*, 2015). Phylogenetic reconstructions revealed different evolutionary histories for CCR rhizobia compared to their counterparts elsewhere in the world (Dludlu, Chimphango, Stirton, *et al.*, 2018a). For example, *Paraburkholderia* has been identified as the ancestral rhizobial symbiont of CCR legumes (Dludlu *et al.*, 2018a; Sprent *et al.*, 2017) and their exceptional diversity has led to the region being classified as a *Paraburkholderia* biodiversity hotspot (Gyaneshwar, Hirsch, Moulin, *et al.*, 2011; Lemaire *et al.*, 2015). CCR legume-rhizobium associations also tend to differ in terms of specificity. For example, the tribe Psoraleeae tends to preponderantly associate with *Mesorhizobium* strains, while members of the Podalyrieae associate primarily with *Paraburkholderia* strains. Contrastingly, tribes like Crotalarieae and Indigofereae are promiscuous and associate with numerous rhizobial genera (Lemaire *et al.*, 2015). Lastly, *Bradyrhizobium* strains are not frequently associated with native CCR legumes (Lemaire *et al.*, 2015; Le Roux *et al.*, 2016). Therefore, the *Bradyrhizobium*-enrichment which often accompanies acacia invasions (e.g. Kamutando, Vikram, Kamgan-Nkuekam, *et al.*, 2017; Le Roux, Ellis, van Zyl, *et al.*, 2018) may amplify the already severe impacts that these invaders have on native plants (Keller, 2014), through the homogenization of the rhizobial community and the subsequent disruption of effective native associations. Furthermore, as *Bradyrhizobium* has often been co-introduced with acacias into South Africa (Ndlovu *et al.*, 2013; Le Roux *et al.*, 2016; Warrington *et al.*, 2019), there is the possibility for direct competition between exotic and native rhizobia for legume associations. This will result in stronger positive-feedback mechanisms under rhizobial cointroductions compared to when

acacias form novel associations with resident South African rhizobium strains (Le Roux *et al.*, 2017).

Aims and objectives of this study

Invasive Australian acacias are largely classified as promiscuous hosts capable of forming both novel and familiar rhizobial associations in their invaded ranges, including within the CCR (Ndlovu *et al.*, 2013). While associations with rhizobia contribute to their establishment success, it remains unclear how different pathways of mutualist acquisition (i.e. co-introduced familiar associations vs novel associations) may influence acacia colonization and establishment in novel CCR environments and the concomitant impacts of the presence/absence of exotic rhizobia on native species. Additionally, rhizobia community composition is largely determined by soil characteristics, such as soil pH (Dludlu *et al.*, 2018a). As such, different soils may harbour different rhizobial strains as well as be more conducive to exotic rhizobial survival. Therefore, Chapter 2 addresses the aims of determining i) whether familiar rhizobial associations facilitate acacia growth performance (as a proxy for acacia colonization success) in pristine CCR soils where acacia congenerics are absent and ii) how the growth of a native legume may be affected under similar circumstances.

Within the CCR, Australian acacias are the most damaging invaders due to strong positive feedback mechanisms (Gaertner *et al.*, 2009). These feedbacks are predominantly driven by the high leaf-litter input, a result of the rapid growth rates and biomass accumulation of acacias. This, in turn, often results in changes in abiotic soil conditions, such as decreased pH, increased soil nitrogen and moisture levels, as well as increased concentrations of allelopathic chemicals (Yelenik *et al.*, 2004). These impacts facilitate acacia establishment and survival, simultaneously negatively impacting on native species. *Acacia*-induced soil changes have also been found to benefit acacia nodulation by their preferred *Bradyrhizobium* partners (Le Roux *et al.*, 2018). Moreover, some acacias have been cointroduced into the CCR with their preferred *Bradyrhizobium* strains (Ndlovu *et al.*, 2013; Warrington *et al.*, 2019). Altogether, numerous mechanisms may be at play to increase the competitive ability of acacias over native plants. However, the relative roles of positive feedbacks and cointroduction of rhizobia in driving the competitiveness of acacias is yet to be teased apart. Therefore, Chapter 3 aims to assess i) the relative contributions of the positive-feedback mechanisms generated by acacia leaf litter and the presence of familiar rhizobial associations, towards their

competitiveness and ii) whether acacia-mediated positive feedbacks and the presence of exotic rhizobial strains (i.e. novel associations) negatively impact the competitive ability of a native legume.

Figures

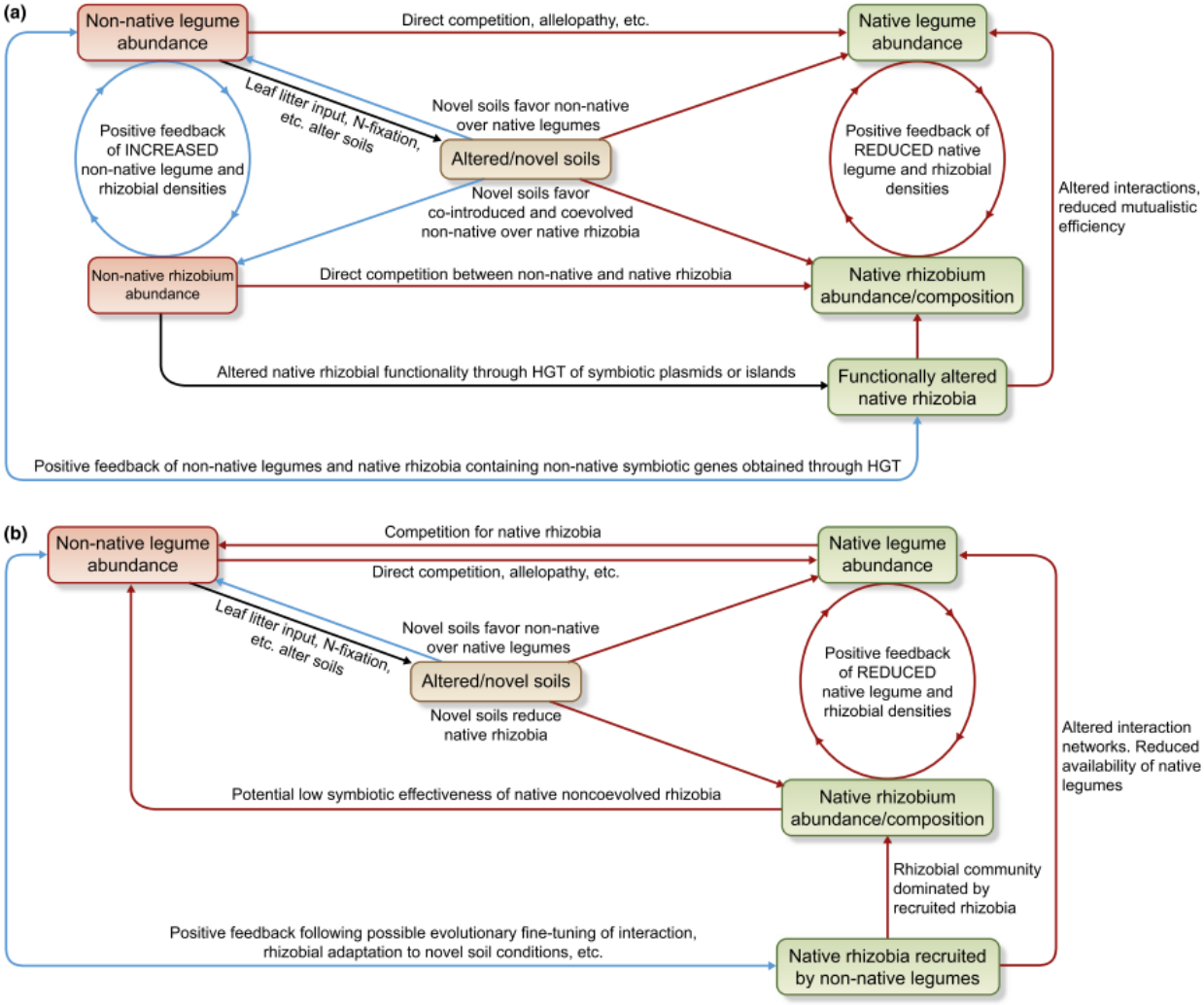


***Figure 1.1**: Using the association between legumes and rhizobia as an example, there are various feedback mechanisms and associated impacts of non-native legumes on native legumes and their associated rhizobia under the two pathways of mutualist acquisition: (a) familiar associations through rhizobial cointroductions and (b) novel associations with resident rhizobia. Blue and red arrows indicate direct positive negative effects, respectively. Black arrows indicate the processes through which non-native legumes alter soils and native rhizobial functionality during invasion, thus resulting in indirect impacts (taken with permission from Le Roux* et al., *2017).*

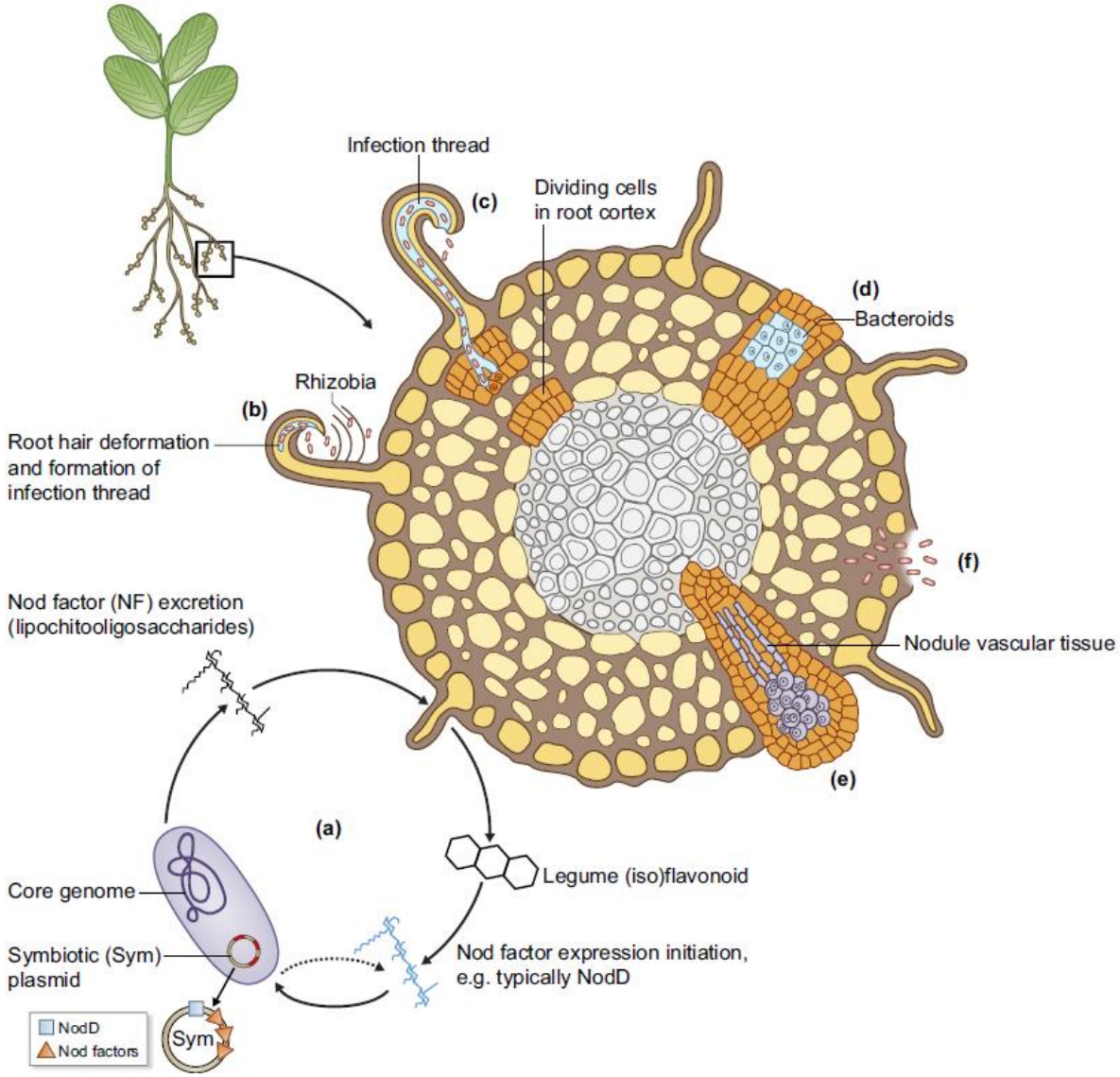


***Figure 1.2**: An illustration of the process of nodulation where exudates from the legume stimulate the activation of the* nodD *gene which is followed by the cascading expression of the* nodABC *gene complex to produce Nod factors (a). Nod factors, in turn, trigger various responses in the legume which results in root hair re-orientation (b) and rhizobial entrapment via the infection thread (c), ultimately leading to nodule formation (d; e). Some rhizobia have evolved to bypass this plant-microbe molecular communication by entering the host plant via crack in the epidermis (f) (taken with permission from Le Roux* et al.*, 2017).*

Chapter 2

The effects of exotic rhizobia on the performance of an invasive and native legume in pristine fynbos soils.

Abstract

Mutualisms are often vital for plant survival and are disrupted when non-native plants are introduced to novel environments. The absence of effective mutualisms in novel environments may act as a barrier for the successful establishment of non-native plants. However, many non-native species maintain mutualistic associations by forming i) novel associations with native resident partners or ii) familiar associations with cointroduced partners. Invasive Australian acacias are known to have reassembled nitrogen-fixing rhizobium mutualisms through both pathways. Familiar associations are expected to cause higher impact severity on native species. This chapter examines the contributions of novel vs familiar rhizobial associations to *Acacia saligna* growth performances as a proxy of colonization success across different soils within the Core Cape Subregion (CCR) and the concomitant impacts of co-introduced rhizobia on a native legume, *Psoralea pinnata*. I grew each species separately in a glasshouse experiment and in different pristine CCR soils and subjected them to Australian bradyrhizobia inoculum treatments. Various seedling performance measures were recorded and next-generation sequencing (NGS) barcoding was used to identify rhizobia associating with each species. Overall, I found the presence of Australian bradyrhizobium to rarely affect the performances of both species while different soil types often impacted growth performances. NGS barcoding revealed that, regardless of inoculum treatment or soil type, each species associated with their preferred (and familiar) rhizobial partners. That is, *A. saligna* associated predominantly with Australian *Bradyrhizobium* strains and *P. pinnata* with native CCR *Mesorhizobium* strains. This suggests that Australian bradyrhizobia are already present and widespread in pristine CCR soils. Consequently, the presence of familiar and effective rhizobia may facilitate the establishment of introduced Australian acacias within the CCR. Additionally, the ability of *P. pinnata* to sanction exotic bradyrhizobia, and the apparent co-existence between these strains and *Mesorhizobium*, suggests that *P. pinnata* may be a good candidate for active restoration projects.

Introduction

Novel (a)biotic conditions can act as barriers to the successful establishment of introduced non-native species (Blackburn, Pyšek, Bacher, *et al.*, 2011). Many plants rely on mutualists for successful reproduction (e.g. pollination), dispersal (e.g. myrmecochory) and nutrient acquisition (e.g. mychorrization), however, these mutualisms are often disrupted during introduction into new environments (Richardson *et al.*, 2000; Parker, 2001). The re-establishment/replacement of effective mutualisms in the new range depends on the availability and diversity of resident mutualists, as well as the level of interaction specificity of both the introduced plant and resident mutualists. When non-native plants have generalist mutualist requirements, they could form novel and effective associations with (usually generalist) resident mutualists. On the other hand, specialist non-native plants may only persist if their historical (or very similar) associations are maintained (Rodríguez-Echeverría *et al.*, 2011). This can happen when they are co-introduced with their mutualists (i.e. so-called familiar associations; (Le Roux *et al.*, 2017) or when they encounter resident mutualists that are phylogenetically similar to their original mutualists. For example, many pine species (family Pinaceae) introductions initially failed in Southern Hemisphere countries due to a lack of compatible ectomychorrizal fungal partners (Policelli *et al.*, 2019). It was only after the introduction of these mutualists that pines established and became widespread invaders in these countries (Richardson *et al.*, 1994). In the absence of cointroduction, novel associations would require some selection and fine-tuning of compatible resident mutualists, while the maintenance of familiar associations will be largely dependent upon the successful survival of co-introduced mutualists in the new environment (Le Roux *et al.*, 2017).

The legume family (Fabaceae) comprises approximately 19,500 species. Many legumes form mutualistic associations with nitrogen-fixing soil bacteria, known as rhizobia. These bacteria form nodules on the roots and, less commonly, the stems of their hosts. Within these nodules, rhizobia fix atmospheric nitrogen into forms that their legume hosts can utilize in return for carbon-rich photosynthates. Legumes are also often over-represented in alien floras, with approximately 1,189 naturalized species globally, including symbiotic nitrogen-fixing and non-symbiotic taxa (Pyšek, Pergl, Essl, *et al.*, 2017). It appears that range expansion by symbiotic non-native legumes is constrained by the availability of effective rhizobial symbionts (Simonsen *et al.*, 2017), with generalist legumes being more likely to become widespread than those with specialist rhizobial requirements (Harrison *et al.*, 2018; Klock *et*

al., 2015). Highly invasive legumes, therefore, often form associations with different rhizobia in their native and non-native ranges (e.g. Australian *Acacia* spp., *Cytisus* spp., *Leucaena* spp. and *Robinia* spp. in Brazil – de Faria & de Lima, 1998; *Acacia pycnantha* in South Africa – Ndlovu *et al.*, 2013; *Trifolium* spp. in New Zealand – Shelby *et al.*, 2016). On the other hand, specialist legumes usually fail to colonize new areas when they are not co-introduced with their co-evolved rhizobia. The link between symbiotic promiscuity, cointroduction, and invasiveness is elegantly illustrated by the globally invasive legume genus *Mimosa*. In India, *Mimosa pudica* was unable to effectively associate with rhizobial strains associated with co-occuring native *Mimosa* species and only successfully established following the introduction of its familiar rhizobial mutualist from South America (Gehlot *et al.*, 2013; Melkonian *et al.*, 2014).

While highly invasive legumes are expected to be promiscuous, cointroductions of non-native legumes and their rhizobia appear to be commonplace (*Cytisus scoparius* in North America (Horn *et al.*, 2014); *Mimosa* spp. in Australia (Parker *et al.*, 2007), China (Liu *et al.*, 2012), India (Gehlot *et al.*, 2013), and Taiwan (Chen *et al.*, 2005)). Some legume groups have been repeatedly found to have been co-introduced with their rhizobia. For instance, Australian acacias (genus *Acacia* Mill.) and their rhizobia have been co-introduced to South Africa (Ndlovu *et al.*, 2013; Le Roux *et al.*, 2016; Warrington *et al.*, 2019), New Zealand (Warrington *et al.*, 2019; Weir *et al.*, 2004) and Portugal (Crisóstomo *et al.*, 2013; Valdovinos, Ramos-Jiliberto, Garay-Narváez, *et al.*, 2010) and to their non-native ranges in Australia (Birnbaum, Bissett, Thrall, *et al.*, 2016). In places like South Africa, different acacia species show variable invasiveness based on geographic spread. Yet, Keet *et al.* (2017) recently found that widespread and localized acacia species associate with only one or two co-introduced *Bradyrhizobium* strains. Acacias in South Africa are also known to form novel associations with resident CCR rhizobia (e.g. Ndlovu *et al.*, 2013). These examples illustrate that acacias are promiscuous host plants capable of forming novel (i.e. with resident rhizobia) and maintaining familiar (i.e. with co-introduced rhizobia) associations in their new ranges.

South Africa's Core Cape Subregion (CCR) (Manning & Goldblatt, 2012), is renowned for its exceptional plant diversity, attributed, in part, to a complex mosaic of soil conditions (Cowling, Procheş & Partridge, 2009; Linder, 2003, 2005). The region is home to an estimated 764 native legumes (Manning & Goldblatt, 2012). Unsurprsingly, the CCR is also a hub for

exceptionally high and endemic rhizobial diversity, with all major genera, including *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* (*alpha*-Proteobacteria), and *Paraburkholderia* (formerly *Burkholderia* (Sawana *et al.*, 2014); *beta*-Proteobacteria), found in the region (Beukes *et al.*, 2013; Elliott *et al.*, 2007; Gerding *et al.*, 2012; Hassen *et al.*, 2012; Kanu & Dakora, 2012; Kock, 2004; Lemaire *et al.*, 2015; du Preez, 2019). Heterogenous soil conditions in the CCR are also perceived as important in determining the composition of the aboveground legume community (Dludlu, Chimphango, Stirton, *et al.*, 2018b), and, in turn, the composition of native rhizobial communities (Dludlu *et al.*, 2018a; Keet *et al.*, 2017; Lemaire *et al.*, 2015). Some rhizobial genera, like *Paraburkholderia*, are known to exhibit edaphic specialisation, with members often being restricted to low pH soils (Dludlu *et al.*, 2018a). Others (e.g. *Bradyrhizobium* – Rodríguez-Echeverría, Pérez-Fernández, Vlaar, *et al.*, 2003; and *Mesorhizobium* – Dludlu *et al.*, 2018a) have relatively cosmopolitan distributions and are less sensitive to high edaphic variation.

While Australian acacias are promiscuous hosts, they exhibit a clear preference for *Bradyrhizobium* strains in both their native (Birnbaum *et al.*, 2016; Lafay & Burdon, 2001; Lange, 1961) and non-native ranges (Kamutando, Vikram, Kamgan-Nkuekam, *et al.*, 2019; Keet *et al.*, 2017; Le Roux *et al.*, 2016). Bradyrhizobia are not common associates of native CCR legumes, and are usually infrequently found in soils and at low abundances (Lemaire *et al.*, 2015). However, their low sensitivity to fluctuations in soil pH, coupled with their cosmopolitan distribution, may benefit *Bradyrhizobium* specialists like introduced Australian acacias. As mentioned, acacias and Australian bradyrhizobia have been co-introduced to South Africa (Ndlovu *et al.*, 2013; Warrington *et al.*, 2019). The low sensitivity to edaphic conditions inherent of *Bradyrhizobium* and the presense of a compatible hosts may, therefore, facilitate both the survival of exotic *Bradyrhizobium* strains and, subsequently, the successful colonization by introduced acacias. Indeed, previous studies have found acacia invasion to result in localized enrichment of *Bradyrhizobium* strains in the CCR (Kamutando *et al.*, 2019; Keet *et al.*, 2017; Le Roux *et al.*, 2018; Slabbert, Jacobs & Jacobs, 2014). Over larger spatial scales such enrichment can lead to homogenization of rhizobial communities and lower native rhizobial diversity (Kamutando *et al.*, 2019; Le Roux *et al.*, 2018; Weir *et al.*, 2004). This, coupled with the incompatibility between CCR legumes and Australian bradyrhizobia, may have negative consequences for native legumes. In Portugal, for example, it has been shown that co-introduced bradyrhizobia outcompete native rhizobia and form less effective symbioses

with native legumes (Rodríguez-Echeverría, Fajardo, Ruiz-Díez, *et al.*, 2012). Moreover, because acacias can utilize the same bradyrhizobia interchangeably, invasive populations may facilitate the successful colonization of congeners, a phenomonan known as invasional meltdown (Keet *et al.*, 2017; Warrington *et al.*, 2019).

Despite the wealth of information on acacias and their rhizobia in the CCR, it remains unclear how the presence of Australian rhizobia affect the growth performance of invasive acacias and co-occurring CCR legumes. Here I aimed to address this question. A glasshouse experiment was used to compare the performance of invasive *Acacia saligna* and native *Psoralea pinnata* grown in different CCR soil types, with or without the presence of Australian *Bradyrhizobium* strains. Next generation sequencing approaches were used to characterize the root nodule communities of both legumes under these different treatments. I hypothesised that the performance of *A. saligna* would be enhanced when forming familiar associations under inoculum treatments while the performance of *P. pinnata* would be negatively impacted.

Methods

Study system

Acacia saligna (Labill.) Wendl., commonly known as Port Jackson willow, is native to Western Australia and is invasive in many countries across the globe. Of the 15 invasive Australian acacias present in South Africa, *A. saligna* has the fifth largest distribution (Richardson, Le Roux & Wilson, 2015) and is classified as a category 1b invasive according to the National Environmental Management: Biodiversity Act (Act 10 of 2004) as listed under section 70(1)(a). In South Africa, the species forms dense thickets that have had many devastating impacts on above- and belowground biodiversity and edaphic characteristics (Le Maitre *et al.*, 2011). For instance, their high leaf litter production, coupled with their ability to fix atmospheric nitrogen, leads to nitrogen enrichment in the usually nutrient-poor soils of the region (Le Roux *et al.*, 2018; Yelenik *et al.*, 2004). *Acacia saligna* is promiscuous, associating with many rhizobial species of both the *alpha*- and *beta*-Proteobacteria, but, like most Australian acacias, has a preference for *Bradyrhizobium* strains (Keet *et al.*, 2017; Lafay & Burdon, 2001; Marsudi *et al.*, 1999).

Psoralea pinnata L., commonly known as fountain bush, is native to the CCR and is a member of the Papilionoid subfamily of the Fabaceae. It occurs across the Cape Peninsula to

the Kogelberg within a variety of fynbos vegetation types, particularly on acidic, nutrient-poor sandstone-derived soils or in richer shale soils (Bello, Stirton, Chimphango, *et al.*, 2017). Previous studies have found *P. pinnata* to be predominantly nodulated by *Mesorhizobium* strains (Kanu & Dakora, 2012; Lemaire *et al.*, 2015). However, associations with *Paraburkholderia* spp. and *Rhizobium* spp. have also been documented (Kanu & Dakora, 2012). Interestingly, *P. pinnata* has been introduced to western and eastern Australia where it has become naturalized and has been identified as a potential future invader, including in habitats where *A. saligna* naturally occurs (Stirton, Stajsic & Bello, 2015). *Psoralea pinnata* is frequently found growing in sympatry with acacias in the CCR (personal observation).

Soil collection

Five different soils were collected from pristine CCR areas with the aim of capturing a range of abiotic (edaphic characteristics) and biotic (rhizobial enrichment by *P. pinnata*) conditions. These soils were collected across the Stellenbosch Winelands and Overberg districts of the CCR (see Table S2.1 for site details) in October 2018.

Four of these soils were collected at sites where neither *P. pinnata* nor *A. saligna* were present. These sites were located in the Grootbos Private Nature Reserve, Kogelberg Nature Reserve, Rustenberg Winery, and Vergelegen Wine Farm. At each site, soils were collected from four locations that were approximately 5m apart. The topsoil (approximately the first 5cm of soil) was scooped aside and approximately 25L of soil were excavated at each location. These were then mixed for each site and stored within a sterile 110L opaque plastic storage container to make up a total of 100L of soil for each site. All soil sampling equipment was rinsed and sterilized with 70% ethanol between collections.

A fifth soil type, hereafter referred to as *Psoralea*-conditioned soils, was collected from directly under five individual *P. pinnata* shrubs spread across three different sites: Prawn Lake in Hermanus, Kogelberg Nature Reserve and Vergelegen Wine Farm. Shrubs chosen within the same site were a minimum of 50m apart from one another. All *P. pinnata* shrubs chosen were over 1.5m tall and were part of a well-established *P. pinnata* population. The excavation procedure was the same as for the other four soils. Twenty liters of soil were collected from within a 1m radius of each of the five shrubs and bulked, and thoroughly mixed, to make up 100L of soil in total. This was stored in a 110L sterile opaque plastic container.

At the end of the collection period, all soils were separately sieved through a 4mm mesh in order to remove any plant debris and rocks. The sieve and all equipment were sterilized with 70% ethanol between sieving of individual soils. Soils were then returned to storage containers and stored at room temperature for a period of three months before commencing with the glasshouse experiment.

Glasshouse experimental setup

For the glasshouse experiment, a layer of unsterilized drainage chips followed by two litres of the collected site-specific soil were placed into green plastic gardening pots (18cm diameter x 15.5cm height) which were each placed onto a water collecting saucer (20cm diameter). This was done for a total of 40 pots per soil type (five soil type; total n = 200). Equipment used during this process was sterilized with 70% ethanol between potting the different soil types. All pots were then watered with tap water until soils were water-saturated.

Seeds of *A. saligna*, collected from invasive populations within the Western Cape, were obtained from the Agricultural Research Centre's Plant Protection Research Institute (ARC-PPRI) in Stellenbosch. *Psoralea pinnata* seeds, collected from populations across the Cape Peninsula, were supplied by Silverhill Seeds in Kenilworth, Cape Town. All seeds were surface-sterilized and scarified prior to planting. Surface sterilization was done by submersion in 90% ethanol for 1min followed by submersion in a 6% bleach solution for 5min, followed by three rinses in distilled water (Birnbaum *et al.*, 2012). *Psoralea pinnata* seeds were scarified by soaking in 60°C sterilized distilled water (dH_2O) (Siva, Sivakumar, Premkumar, *et al.*, 2014) and *A. saligna* seeds were scarified by nicking a portion of the seed coat to expose the endosperm followed by soaking in luke-warm dH_2O (Rincón-Rosales, Culebro-Espinosa, Gutierrez-Miceli, *et al.*, 2003). Seeds of both species were soaked for one hour. Four seeds of *A. saligna* were then planted into each of 20 of the pots per soil type. The same was done for *P. pinnata* seeds for the remaining 20 pots per soil type. Seeds were allowed to germinate and the seedlings to establish for a given period of five weeks. After this five week period, all but one were haphazardly removed from pots when more than one seed germinated per pot. In a few pots, none of the seeds germinated. To make up for these losses, extra seedlings removed from pots with high germination success were transplanted into these pots, within the same species x site x inoculum treatment combinations.

As a means to ensure that rhizobial communities were still present within the soils post-storage, fresh soil was collected from each site and applied to the pots as a soil inoculum (van de Voorde, van der Putten & Bezemer, 2012). Soil collections were done according to the same protocol as for the previous soil collections and preparations, except only a total of 60L of each soil type was collected. Six weeks post-planting, 0.2L of this fresh soil was added to the soil-specific pots (i.e. each pot containing a specific soil type received soil inoculum of the same type) for both species and care was taken not to smother the seedling in the process. All equipment was sterilized with 70% ethanol between additions of the soil from the different sites.

Australian rhizobial inoculum preparation

After one week following the addition of soil inoculum an Australian rhizobium inoculum was applied (i.e. 7 weeks post-planting). For inoculum preparation, five strains of *Bradyrhizobium* previously isolated from *Acacia dealbata*, *A. decurrens* and *A. melanoxylon* in Australia were used. Although these strains were not isolated from *A. saligna*, they are of Australian origin and previous work has illustrated that acacias interchangeably use the same bradyrhizobia with similar efficacy (Keet *et al.*, 2017; Wandrag, Sheppard, Duncan, *et al.*, 2013; Warrington *et al.*, 2019). These strains were grown separately in a Yeast Mannitol liquid broth through shake incubation (155rpm) at 28°C for a period of 5 days. Fifteen millilitres of each strain were mixed together, creating a rhizobial cocktail (75mL) which was diluted in 1425mL dH_2O to make up a total inoculum volumn of 1.5L. Using a pipette, 5mL of this Australian rhizobial cocktail was added as an inoculum to 10 of the 20 pots per species per soil (n = 10 for each species x soil type x inoculum addition combination). The remaining 10 pots for each soil type received 5mL of sterile Yeast Mannitol broth that had been diluted in the same manner as the inoculum. Inoculum addition was repeated four weeks later.

Glasshouse experiment protocols and measurements

Prior to the addition of the rhizobial cocktail, pots were watered every second day with tap water using a watering can. In order to minimize cross-contamination of the added rhizobium inoculum, a more stringent watering system was put in place whereby each pot was individually watered every two days with all pots receiving the same volume of water. Plants were grown for a total of 17 weeks from day of planting (February 2019) to harvest (June 2019)

as the seedling stage is particularly crucial for establishing effective mutualistic associations (Parker, 2001). This 17-week period can be separated into an initial 5-week period for germination and seedling establishment, and a 12-week period during which seedlings were exposed to inoculum treatments. Throughout the growth period, pots were kept in a glasshouse exposed to ambient light and temperature conditions. All pots were randomly placed within the glasshouse and randomized weekly to reduce the potential effects of microclimates within the glasshouse on seedling growth. All randomization took place prior to watering when saucers were dry in order to minimize any potential cross-contamination through spillage.

Seedling height (defined as the length between the point where the stem exits the soil surface and the furthest apical meristem from this point along the main stem) was measured using a 30cm ruler. To harvest seedlings with minimal damage to their root systems, pots were vigorously tapped to loosen the soil. The seedling and the pot were then inverted and the pot was removed. Soil was then carefully shaken off the roots so that a negligible number of nodules were lost during the process. All excess soil was rinsed off by dunking the root system into a bucket of tap water and the roots were dabbed dry using tissue paper.

All root nodules for each seedling were removed from roots, counted and placed into tubes containing silica crystals to desiccate them. This was done separately for each seedling. Finally, seedlings were cut at the point where stems transitioned to roots (identified using the colour change from green to brown) and the shoots and roots were placed into separate brown paper bags. At the end of harvest, all bagged plant material was placed into a drying oven at 55°C for one week. All dried plant material was weighed to determine root (excluding nodules) and shoot dry biomass, total dry biomass and root:shoot biomass ratios. These measurements, together with measurements of seedling height, were used as indicators of plant growth performances.

All desiccated nodules were weighed for each plant separately. As a measure of biological nitrogen fixation (BNF) efficiency, I also measured δ^{15}N isotopic signatures. Phyllode (*A. saligna*) or leaf (*P. pinnata*) material was removed after weighing of shoot biomass. The material was crushed into a fine powder using a tissuelyzer and processed according to the guidelines specified by iThemba Labs, Pretoria, for isotopic analysis. Briefly, this entailed weighing out between 0.5 to 0.8mg of the crushed leaf powder in a 1cm by 0.5cm

tinfoil cup which was then folded into a tiny square to seal the powder inside. Each sample was housed within an individual well of a flat-bottomed 96-well plate and sent to iThemba Labs. Isotopic analyses were conducted using a Flash HT Plus integrated via a ConFlo IV system with a Delta V Plus Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). Samples were combusted at 1,020°C and the nitrogen isotope values corrected against an in-house standard (Merck Gel $\delta^{15}N$ = +6.80‰). Isotope values are expressed in parts per thousand (‰) following Lötter, Archer Van Garderen, Tadross, *et al.* (2014) and Rodríguez-Echeverría, Crisóstomo, Nabais, *et al.* (2009):

$$\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000\ ‰$$

where $\delta^{15}N$ is the heavier ^{15}N isotope, and R is the ratio of the heavy ^{15}N to the lighter ^{14}N isotopes for the sample and the standard (i.e. atmospheric nitrogen) respectively. Generally, $\delta^{15}N$ are expressed as parts per thousand deviation from the ^{15}N composition of atmospheric nitrogen (0‰) (Mariotti, 1983). The lighter isotope is incorporated into reactions. Therefore, one would expect that during BNF, ^{14}N would have a higher relative abundance in the resultant NH_3 than ^{15}N. Consequently, NH_3 produced through BNF would have lower or even negative $\delta^{15}N$ values compared to NH_3 produced via non-BNF processes given that atmospheric nitrogen is in unlimited supply (Unkovich, 2013). Therefore, low or negative $\delta^{15}N$ values may be indicative of BNF compared with higher values which suggest that NH_3 was assimilated from soil nutrient pools (Lajtha & Marshall, 1994; Unkovich, 2013). These $\delta^{15}N$ measurements, together with the nodule count and nodule total dry biomass measurements, were used as proxies for BNF.

Statistical analyses of growth performance and BNF measurements

All statistical analyses were conducted in the R statistical environment (v3.4.4 R Core Development Team) and separately for each species.

To investigate the effect of Australian inoculum addition as well as potential effects of each soil's (a)biotic characteristics on growth performance measurements (i.e. seedling height, seedling shoot dry biomass, seedling root dry biomass, seedling total dry biomass, root:shoot ratios) and BNF (i.e. number of nodules, nodule total dry biomass, $\delta^{15}N$), generalized linear mixed models with Guassian distributions (link = "identity") were fitted using the *lme* function

in the *nlme* R package (Pinheiro, Bates, DebRoy, *et al.*, 2013) with Australian inoculum addition (with or without inoculum addition), soil (Grootbos, Kogelberg, Rustenberg, Vergelegen and *Psoralea*-conditioned) and their interaction as fixed effects. A random factor ('transplanted') was included to account for potential differences due to transplanting of seedlings in pots where initial germination had failed. Overall effect sizes and their significance were determined using the *anova* function in the R base package (Type I sum of squares were used as the order of the fixed effects of the model did not alter the outcome). Pairwise contrasts between levels of the fixed effects were determined using the *emmeans* function in the *emmeans* R package (Lenth, Singmann, Love, *et al.*, 2018). Additionally, in order to determine whether the δ^{15}N values for each growth setup by inoculum addition treatment combination was significantly different from zero, I used a one-sample t-test (mu=0) or a one-sample Wilcoxin test (mu=0) for parametric and non-parametric groups, respectively. This was repeated for both species.

In order to gain a clearer understanding of the role of inoculum addition on the encounter rate of rhizobia by the seedlings, data for all soils were combined and the total number of nodules regressed against the seedling root dry biomass. The encounter rate of rhizobia in soils is positively correlated with the amount of root surface area (or root biomass) produced (Ramoneda, Le Roux, Frossard, *et al.*, 2020). Therefore, instead of measuring exact encounter rates, the expected relationship between root biomass and nodule number was investigated in order to determine whether this relationship was influenced by the addition of exotic rhizobia. Nodule number was regressed against seedling root dry biomass using a generalized linear mixed model (Guassian distribution; link = "identity") with the *lme* function in the *nlme* R package (Pinheiro *et al.*, 2013). For *P. pinnata*, nodule number best fit a poisson distribution, therefore, a generalized linear mixed model generated with the *glmer* function in the *lme4* R package (Bates, Mächler, Bolker, *et al.*, 2015; Bates, Maechler, Bolker, *et al.*, 2018) with a poisson distribution (link = "log") was used. In both models, dry root biomass was the continuous predictor variable and Australian inoculum addition was the categorical predictor, and an interaction term between the two was also included. Random effects included 'soil' and 'transplanted', with transplanted nested within soil (i.e. unbalanced design) (Bates *et al.*, 2015). Overall effect sizes and Chi-squared values for *A. saligna* and *P. pinnata*, respectively, and their significance for each main effect was determined using the *Anova* function (type III sum of squares) in the *car* R package which is suitable for unbalanced designs with significant

interaction terms and for extracting overall p-values from generalized linear models (Langsrud, 2003; Macnaughton, 1998). The marginal and conditional R^2 values, which indicate the amount of variance explained by fixed effects only and the model as a whole, respectively, were calculated using the *rsquared* function in the *piecewiseSEM* R package (Nakagawa & Schielzeth, 2013). The amount of variation explained by the random effects (random effects R^2) was determined by calculating the difference between the conditional R^2 and marginal R^2 values (Nakagawa & Schielzeth, 2013).

Similar model structures to those used for encounter rates were also used to determine the relative contribution of nodules to seedling growth performance and BNF under the two inoculum treatments, i.e. the average gain in performance with increased nodulation. It is expected that the efficacy of mutualist associations will increase under familiar associations, and that steeper and positive relationships between nodule numbers and growth performance measures would be evident (Le Roux *et al.*, 2018). Data for all soils were again combined and the different growth performance and BNF measures regressed against nodule number using generalized linear mixed models (*lme* function in *nlme* R package) as all variables best fit a Gaussian distribution (link = "identity"). In this case, fixed effects included total nodule number as the continuous predictor variable, Australian inoculum addition as the categorical predictor, and their interaction. Random effects once again included 'site' and 'transplanted', with transplanted nested within site. The marginal and conditional R^2 values as well as the R^2 values of the random effects were also calculated for each measurement.

DNA extraction and next-generation sequencing (NGS) of root nodule and inoculum rhizobia

In order to determine the rhizobial identity and abundances within root nodules of *A. saligna* and *P. pinnata*, I pooled between 3 and 5 nodules from each seedling within a particular species x soil x inoculum treatment combination for DNA extraction. This was done for each of the 20 species x soil x inoculum treatment combinations (i.e. 20 samples in total). Desiccated nodules were tissuelyzed into a fine powder to create a homogenous mixture of nodule material. DNA was extracted from these homogenous mixtures using the DNeasy® Plant Mini Extraction Kit (Qiagen, supplied by White Head Scientific, Cape Town, South Africa) according to the manufacturer specifications.

To extract DNA of the *Bradyrhizobium* strains used in the inoculum cocktail, all five strains were grown from glycerol stocks separately in Yeast Mannitol broth in a shaking incubator (155rpm) at 28°C until there was sufficient bacterial growth (indicated by a milky turbit colour change). DNA was extracted from cultures using the Sigma Gen-Elute Bacterial Genomic DNA kit (Sigma-Aldrich Co. LLC, USA), according to manufacturer specifications. Isolated DNA concentrations and quality were checked using a NanoDrop ND-1,000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). These samples were used as reference strains in subsequent analyses.

We amplified the nodulation C (*nodC*) gene for NGS, using the primers nodCF12F (5'-CCG GAT AGG MTG GKB CCR TA-3') and nodCRI2R (5'-GTG CAC AAS GCR TAD RCC TTC AH-3'), with sample-specific barcodes in the forward primer. This gene has been successfully utilized for taxonomic identification purposes across rhizobia in both the *alpha*- and *beta*-Proteobacteria (Le Roux *et al.*, 2016).

Amplification was done using a 30-cycle PCR and the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following PCR conditions: 94°C for 3min, followed by 28 cycles of 94°C for 30s, 53°C for 40s and 72°C for 1min, followed by a final elongation at 72°C for 5min. After amplification, PCR products were checked on a 2% agarose gel to determine amplification success and the relative band intensities. Multiple PCR samples (each sample representing the contents of the pooled nodules per species x site x inoculum addition treatment combination) were barcoded first and then pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled PCR samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA) and used to prepare DNA libraries by following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at the Molecular Research LP next-generation sequencing service (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument following manufacturer protocols.

NGS bioinformatics

NodC sequences were joined, and sequences <150bp in length or with ambiguous base calls were removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised; unique sequences

identified with sequencing or PCR point errors were removed, followed by chimera removal, yielding zero-Operational Taxonomic Units (zOTUs).

I clustered zOTUs at 97% DNA sequence similarity via the nearest neighbour algorithm, based on pairwise sequence similarity distances calculated with the Needleman-Wunsch algorithm in mothur v1.44.1 (Schloss, Westcott, Ryabin, *et al.*, 2009). Since no reference database exists for *nodC* sequences, representative sequences of each OTU were blasted against the NCBI's GenBank database (http://blast.ncbi.nlm.nih.gov/Blast) to determine taxonomic identity. All non-nitrogen-fixing bacteria were removed from the dataset so that only rhizobial strains were considered in subsequent analyses.

We found many low-abundance OTUs (often <100 sequence reads/sample). Therefore, the relative abundance of each OTU within a single sample (i.e. host x soil x inoculum treatment combination) was calculated and all rare OTUs, i.e. those making up less than 5% of the cumulative abundance per sample for all samples, were removed from the dataset, resulting in a final dataset comprising 13 OTUs. Because DNA samples pertaining to experiments for both Chapter 2 and 3 were analysed simultaneously, these 13 OTUs make up the entire dataset for both research chapters. There is an overlap of seven OTUs between the two chapters, with three OTUs being specific to Chapter 2 and three OTUs being specific to Chapter 3 (see Results on page 29).

Phylogenetic analysis

Blast results of the 13 OTUs indicated that a large number of OTUs belonged to the genus *Bradyrhizobium*, the preferred symbiont of Australian acacias. In order to determine the geographic origin of the *Bradyrhizobium* strains isolated from my nodules, I obtained additional *nodC* NGS data generated using the same approaches outlined above (i.e the same primers and NGS platform) of *Bradyrhizobium* strains previously collected from acacias (Keet *et al.*, 2017) and acacia-invaded soils in South Africa (Le Roux *et al.*, 2018). I also downloaded *nodC* sequence data from Genbank for bradyrhizobia previously isolated from native CCR legumes (Lemaire and Muasya, unpublished). These additional sequence data were aligned with data for my 13 OTUs using ClustalW in BioEdit (Hall, 1999).

The best-fit nucleotide substitution model for the aligned dataset was determined using JModelTest (Posada, 2008) and Akaike information criterion (Akaike, 1973). The HKY + G + I (Hasegawa, Kishino & Yano, 1985) model was identified as the best fit model. I then used MEGA X (Kumar, Stecher, Li, *et al.*, 2018; Stecher, Tamura & Kumar, 2020) to recontruct a phylogeny using this model and maximum likelihood search criteria. Bootstrap values were calculated using the majority rule consensus method to assess topological support of the phylogeny.

OTU comparisons between treatments

All OTU comparisons between treatments were conducted in the R statistical environment (v3.4.4 R Core Development Team). Importantly, these comparisons were conducted only for the 10 OTUs pertaining to this chapter and excluded those that are specific to Chapter 3.

In order to investigate the prevalence of the Australian inoculum *Bradyrhizobium* strains associating with *A. saligna* seedlings under the two inoculum treatments, the relative abundances of the dominant reference samples' OTUs were compared between the two treatments. These comparisons were only performed for SW OTU1 and SW OTU6 as these were the only OTUs present within the reference samples with a relative abundance >5% (see Results page 30). Relative abundance data were combined for all soils and compared between the two inoculum treatments using a paired t-test and a Wilcoxon signed-rank test for SW OTU1 and SW OTU6, respectively.

The relative abundances of each of the 10 individual OTUs were compared between the different host species x Australian inoculum addition treatment combinations for all soil types combined. This was done in order to determine whether *A. saligna* and *P. pinnata* differed in terms of their rhizobial associations as well as to determine whether these associations differed in the presence of the exotic *Bradyrhizobium* (i.e. inoculum addition). These comparisons were performed using a permutational multivariate analysis of variance (PERMANOVA) in the *vegan* R package. A distance matrix for relative abundance data of all 10 OTUs was developed following the Bray-Curtis dissimilarity method using the *vegdist* function. This distance matrix was used as the response variable in the PERMANOVA and inoculum addition treatment and host species, as well as their interaction, were included as

main effects. The PERMANOVA was run using the *adonis2* function with 999 permutations. Post hoc analyses were performed using the *simper* function in order to elucidate which OTUs were contributing most to any dissimilarities in the nodule rhizobial community composition. All functions form part of the *vegan* R package.

Results

Australian inoculum addition and site interactions

As growth performance measures were frequently significantly correlated, the results shown here are limited to total dry seedling biomass (significantly correlated with root and shoot biomass, and shoot height) and root:shoot ratios. Similarly nodule number (correlated for nodule biomass) and δ^{15}N were used as measures of BNF. Results for all other variables are presented in Supplementary Materials (Tables S2.1 to S2.4; Figs. S2.1 and S2.2).

As expected, there was a significant and a near-significant overall inoculation effect on nodule formation and root:shoot ratios of *A. saligna* seedlings, respectively (Table 2.1). However, this did not translate into significant differences between inoculation treatments within each soil type (Fig. 2.1). A non-significant overall inoculation effect was found for *A. saligna* for total biomass and δ^{15}N values. However, there was a significant interaction between inoculation and soil for these variables (Table 2.1) as shown by the differences in performance between the two inoculation treatments within a particular soil type (Fig. 2.1). This significant interaction also suggests that the direction of the inoculum effect on performances differed between soils. For example, and counterintuitively, this was primarily driven by a reduction in biomass and an increase in δ^{15}N values in *Psoralea*-conditioned soils for those seedlings that received Australian inoculum (Fig. 2.1). Both growth performance and BNF measures differed significantly between soils. In contrast to *A. saligna*, *P. pinnata* growth performances and BNF measures were not influenced by inoculation but varied significantly across soils (Table 2.1 and Fig. 2.1).

In contrast to Australian inoculum addition, soil origin significantly influenced growth performance and BNF of both species (Table 2.1 and Table S2.2). Particularly, both species appeared to have significantly higher total biomass, numbers of nodules and lower δ^{15}N values (i.e. more BNF was occurring) when grown in soils from Rustenberg and *Psoralea*-conditioned soils (Fig. 2.1). These results are more variable for *A. saligna*, with differences between these

two sites and the remaining three sites not being as prominent as they were for *P. pinnata*. Root:shoot ratio responses were largely similar across all five soils for both species, with differences in biomass allocation only manifesting between different inoculum treatments of the same (*A. saligna* in *Psoralea*-conditioned soils) or different (*P. pinnata* in Grootbos, Rustenberg and Vergelegen) soils (see Fig. 2.1 and Table 2.1).

Rhizobial encounter rates

For *A. saligna*, seedling root biomass significantly predicted the number of nodules ($F_{(1, 76)} = 7.411$; $p = 0.008$) (Table 2.2). Intercepts for inoculum treatments were also significantly different ($F_{(1, 76)} = 9.2869$; $p = 0.0032$), with seedlings that received Australian inoculum having a consistently larger number of nodules than those that did not receive inoculum for any given amount of root biomass. However, there was no significant interaction between seedling root biomass and Australian inoculum ($F_{(1, 76)} = 0.2883$; p = ns). Together this indicates that, while those soils receiving Australian inoculum had more rhizobia and the seedlings growing in them had higher nodule numbers, seedlings formed rhizobial associations equally well under either inoculum treatment (Fig. 2.2).

There was a significant interaction between Australian inoculum addition and seedling root biomass for *P. pinnata* ($\chi^2_{(1)} = 12.5765$; $p = 0.0004$), as shown by the steeper slope for those seedlings that received inoculum compared to those that did not (Fig. 2.2; Table 2.2). This suggests that the application of inoculum altered the relationship between root biomass and the number of nodules formed, with seedlings forming associations with rhizobia more readily under inoculum addition as opposed to when inoculum was not applied. However, when one takes the total nodule dry biomass into account (Fig. S2.1), it appears that this increase may be driven by the formation of small, ineffective nodules (particularly in *Psoralea*-conditioned soils) rather than the presence of Australian inoculum facilitating higher encounters with rhizobia. These results need to be interpreted with caution since the overall variance explained by the fixed effects of the model are almost negligible compared to that of the random effects (marginal $R^2 = 0.001$, conditional $R^2 = 0.9914$, R^2 explained by random effects = 0.9904). This suggests that the increase in encounter rate may simply be due to an increase in the abundance of rhizobia within the soils or differences in associations with specific rhizobial genera between soils. For *A. saligna*, however, more of the variance was explained by the fixed effects (marginal $R^2 = 0.1588$, conditional $R^2 = 0.4409$, R^2 explained by random effects = 0.2821).

While the R^2 value for the random effects is still high, it is not as high as for *P. pinnata*, once again highlighting that soil is more important for *P. pinnata* and that the interplay between soil and Australian inoculum addition is important for *A. saligna*.

Rhizobia efficacy

The per capita contribution to symbiosis is an indication of the benefit received by host plants for each nodule formed, i.e. the efficacy of the rhizobial association. For *A. saligna*, there were significant interactions between the number of nodules and Australian inoculum addition for both seedling total dry biomass ($F_{(1, 76)} = 5.7103$; $p = 0.0193$) and for root:shoot ratio ($F_{(1, 76)} = 4.3684$; $p = 0.04$). Seedlings that did not receive inoculum appeared to gain more total biomass and have larger root:shoot ratios for any given number of nodules than those seedlings that did receive inoculum (Fig. 2.3 & S2.2, Table 2.3 & S2.3). This increase in total biomass is likely driven by an overall higher investment in belowground rather than aboveground growth across all soils, as shown by the root:shoot ratios of uninoculated seedlings tending to be higher than those that had received inoculum (Fig. 2.1), although these were non-significant. Additionally, although $\delta^{15}N$ decreased as nodule numbers increased ($F_{(1, 76)} = 9.9139$; $p = 0.0023$) (Table 2.3), there was no significant interaction between Australian inoculum addition and nodule numbers ($F_{(1, 76)} = 3.6837$; p = ns), showing that the presence of inoculum did not increase the BNF/nodule.

Both measures of seedling growth performance and the $\delta^{15}N$ values for *P. pinnata* showed no significant interactions between the number of nodules formed and Australian inoculum addition (Table 2.3). Indeed, upon examining the relative contributions of fixed and random effects in explaining the variances within the models, the conditional R^2 values were once again much higher than the marginal R^2 values. This indicates that the variance explained by the random effects i.e. soil, rather than inoculum may be responsible for the patterns identified for these measures (Table S2.4). This was similar for *A. saligna*, yet there nonetheless appears to be more variance explained by Australian inoculum addition, and less by soil, compared with *P. pinnata* (Table S2.4).

Bioinformatics and phylogeny

After data quality-checking, my *nodC* dataset generated 280 zOTUs. Clustering of these at 97% DNA similarity level, followed by the removal of singleton/doubleton OTUs,

OTUs representing non-fixing bacteria, and OTUs with <5% relative abundance per sample for all samples, resulted in 992,451 sequences representing 13 OTUs for all samples including those specific to Chapter 3 (Table S2.5). Of the 13 OTUs, seven overlapped across both research chapters while three were specific to this chapter, i.e. not present in samples pertaining to Chapter 3 at a relative abundance >5% (SW OTU3; SW OTU17; SW OTU21) and three OTUs were specific to Chapter 3 (SW OTU9; SW OTU12; SW OTU15). OTUs specific to Chapter 3 were included in the phylogenetic analysis of *Bradyrhizobium nodC* sequences, but were not included in comparisons of relative abundance.

Blast results for these 10 OTUs (943,739 sequences) specific to Chapter 2 indicated that they belonged to the genus *Bradyrhizobium* (5 OTUs), *Mesorhizobium* (4 OTUs) and *Rhizobium* (1 OTU) (Table S2.5). Of these, only two OTUs (SW OTU1 and SW OTU6) were present in the the reference samples used as Australian inoculum with a relative abundance >5%. These blasted to *Bradyrhizobium* sp. CPI240 and *Bradyrhizobium* sp. CPI241, respectively, which were previoulsy isolated from *Acacia* spp. in Australia (Barrett, Zee, Bever, *et al.*, 2016). SW OTU1 and SW OTU2 were overridingly the dominant strains isolated from nodules of *A. saligna* and *P. pinnata* respectively, with blast results identifying SW OTU2 as *Mesorhizobium* sp. 969n9 previously isolated from South African legumes (Lemaire & Muasya, unpublished) (Table S2.5). Blast results also revealed that *A. saligna* and *P. pinnata* had also associated with native CCR *Mesorhizobium* strains (SW OTU17 in Grootbos soils) and Australian *Bradyrhiozbium* strains (SW OTU1 in Vergelegen soils), respectively. These are the only instances of novel associations in this study (Fig. 2.4).

The *Bradyrhizobium nodC* phylogeny yielded many unsupported nodes, likely because of the short DNA reads (313 bp) (Fig. 2.5). However, it provided high support for two distinct clades, one including *Bradyrhizobium* strains previously isolated from native CCR legumes and the other bradyrhizobia from my study and strains previously isolated from acacia-invaded soils (JLR OTUs) (Le Roux *et al.*, 2018) and acacia root nodules (JHK OTUSs) (Keet *et al.*, 2017). Interestingly, there were several of my SW OTUs that clustered with JLR OTUs and JHK OTUs with nodal support between 98-100%. Specifically, the dominant *Bradyrhizobium* OTU found in this study, SW OTU1 clusered with the dominant OTUs found by both Keet *et al.* (2017), JHK OTU1, and Le Roux *et al.* (2018), JLR OTU1, with a nodal support of 99%. The second most abundant *Bradyrhizobium* OTU, SW OTU6, clustered with the second most abundant OTU for Keet *et al.* (2017), JHK OTU2 with a nodal support of 98%. SW OTU22

clustered with JHK OTU3 and JLR OTU4 with a nodal support of 99%. Finally, SW OTU12 clustered with JHK OTU25, and SW OTU15 clustered with JHK OTU13, both with a nodal support of 100% (Fig. 2.5).

Root nodule rhizobial composition comparisons

The relative abundances of the two dominant OTUs, SW OTU1 (259,830 sequence reads) and SW OTU6 (10,540 sequence reads), found in the reference samples were similar in *A. saligna* nodules between the two inoculum treatments (SW OTU1: Paired t-test, $t_{(5)}$ = 1.0336, p = ns; SW OTU6: Wilcoxon signed-rank test, W = 11; p = ns).

PERMANOVA indicated that inoculum addition did not significantly change the relative composition of nodule OTU communities ($F_{(1,16)}$ = 0.4052; p = ns). However, the composition of nodule OTU communities differed significantly between host species ($F_{(1,16)}$ = 21.4853, p = 0.001) (Table S2.6). Post-hoc analysis using the *simper* function showed that this significant host species effect was largely driven by SW OTU1 and SW OTU2 which accounted for 35.35% and 34.01% of the total compositional dissimilarity in nodule rhizobial communities between legume species, respectively. This is due to *A. saligna* associating predominantly with *Bradyrhizobium* SW OTU1 while *P. pinnata* predominantly associated with *Mesorhizobium* SW OTU2. The remaining OTUs each accounted for less than 10% of the dissimilarity (Fig. 2.4; Table S2.7).

Discussion

I reject my hypothesis that the presence of exotic Australian rhizobia will negatively impact the native CCR legume, *Psoralea pinnata*, via ineffective novel associations. Whether the opposite is true for invasive Australian *Acacia saligna* (i.e. increased performance due to familiar associations) cannot be wholly resolved as Australian bradyrhizobia appear to already be pervasive in pristine uninvaded CCR soils with only one instance of a novel association for for this species. In fact, *P. pinnata* and *A. saligna* seemed to associate selectively with very different subsets of available rhizobia. That is, *Bradyrhizobium* strains predominantly nodulated *A. saligna*, while *Mesorhizobium* strains predominantly nodulated *P. pinnata*. Importantly, these differences in associations were evident, in most cases, irrespective of inoculum treatment or the soil type in which the seedlings grew. This suggests that the addition of exotic Australian *Bradyrhizobium* strains likely had a limited effect on *P. pinnata*

performance due to the ability of this species to successfully sanction these bradyrhizobia. For *A. saligna*, the fact that nodules from both the inoculated and uninoculated rhizobial treatments contained identical bacterial strains suggests that the limited effects of inoculum addition on performance is likely due to the previously-documented widespread presence of exotic Australian *Bradyrhizobium* in acacia-invaded CCR soils (Keet *et al.*, 2017; Ndlovu *et al.*, 2013; Le Roux *et al.*, 2018; Warrington *et al.*, 2019), although, this is one of the few records of Australian bradyrhizobia in pristine (i.e. uninvaded) CCR soils.

The notion that CCR soils harbour the preferred rhizobia of both *A. saligna* and *P. pinnata* is probably best illustrated by the presence of *Bradyrhizobium* and *Mesorhizobium* strains in all soils collected from pristine sites where neither host was present. My NGS results confirmed *Mesorhizobium* as the preferred symbionts of *Psoralea* spp. (similarly to Kanu & Dakora, 2012; Lemaire *et al.*, 2015). This strong host preference was further illustrated by the high genetic similarity between my dominant *P. pinnata* SW OTU2 and a *Mesorhizobium* strain previously isolated from *P. fleta* in the CCR (Lemaire & Muasya, unpubished). Strong differences in rhizobium associations between acacias and native legumes have also been found previously. For instance, a network analysis by Le Roux *et al.* (2016) found native CCR legumes and invasive acacias to interact with distinct rhizobial assemblages, which these authors argued was due to phylogenetic uniqueness of these host plant groups. They also found that specialised native legumes appeared unable to persist in acacia-invaded areas, whereas generalist legumes could, but only in association with different rhizobia. Our data suggest that the preferred *Mesorhizobium* symbionts of *P. pinnata* can co-exist with Australian bradyrhizobia in CCR soils. In fact, both *Bradyrhizobium* and *Mesorhizobium* strains show similar adaptations to seasonally dry, acidic soils, likely resulting in overlapping distributions (Dludlu *et al.*, 2018a; Rodríguez-Echeverría *et al.*, 2003). Furthermore, *P. pinnata* are often one of the few native CCR legumes to regenerate through passive restoration in sites cleared of Australian acacias, highlighting their ability to survive in *Bradyrhizobium*-enriched soils (Reinecke, Pigot & King, 2008). Legumes are known to minimize the impact of ineffective rhizobial associations through partner choice (i.e. discriminating against ineffective rhizobia during nodule formation) (Heath & Tiffin, 2009; Sachs, Russell, Lii, *et al.*, 2010) and/or through sanctioning (i.e. reducing the oxygen and photosynthate supply to nodules harbouring ineffective rhizobia) (Kiers *et al.*, 2003; Sachs & Simms, 2006). While nodulation by *Paraburkholderia* and *Rhizobium* strains in *Psoralea* spp. have been previously identified

(Kanu & Dakora, 2012; Lemaire *et al.*, 2015), my results suggest that, in most cases, *P. pinnata* can successfully limit associations with exotic *Bradyrhizobium* in favor of their preferred symbionts. Therefore, the impact of co-invading acacias and rhizobia are likely to be negligible on this native legume, at least from a nitrogen-fixing symbiosis perspective.

Regardless of sanctioning, *A. saligna* and *P. pinnata* displayed similarities in terms of the relative abundances of rarer rhizobium OTUs, as indicated by the low percentage contribution of these SW OTUs to the overall dissimilarity of nodule rhizobial community composition between the two species (Table S2.7). Recent evidence suggests that nodule communities are largely made up of a so-called core microbiome, consisting of the preferred symbionts of the host, and a less-dominant/rare group of symbionts (Rodríguez-Valdecantos, Manzano, Sánchez, *et al.*, 2017; Shade & Handelsman, 2012). Furthermore, the means of community assembly of these core and rare strains are often driven by different mechanisms, such as host selection coupled with neutral processes (e.g. drift) and neutral processes alone, respectively (Ramoneda *et al.*, 2020). Although these mechanisms were not explicitly tested, the results of the PERMANOVA and the calculated percentage contribution of each SW OTU to nodule rhizobial community composition dissimilarity suggest that *Bradyrhizobium* and *Mesorhizobium* make up the core symbionts (through host selection) of *A. saligna* and *P. pinnata*, respectively, while the similarities in relative abundances of the rare SW OTUs are likely driven by neutral processes. One such neutral process is encounter probability (Ramoneda *et al.*, 2020). That is, larger root systems may increase the chances of mutualist encounters and, therefore, legume-rhizobium associations. We found rhizobial encounter rates to be higher in *P. pinnata* seedlings that received Australian inoculum compared to those that did not (Fig. 2.2). This observation was mainly driven by the significant increase in nodule number under the Australian inoculum addition treatment in Rustenberg soils (Fig. 2.1). However, this increase in nodule number was not matched by a concurrent increase in total nodule dry biomass (Fig. S2.1), and therefore increased encounter rate may simply reflect an increase in the number of small, ineffective nodules harbouring less-effective rare strains (Kiers *et al.*, 2003).

While it is likely that the relative abundances of the rare OTUs did not differ between host species or inoculum treatments, the association of some of these OTUs with specific sites is worth noting. For example, *A. saligna* associated with a non-dominant OTU in Grootbos

soils, that is, there were high relative abundances of SW OTU17 for these seedlings in both inoculum treatments (Fig. 2.5). Blast results identified SW OTU17 as *Mesorhizobium* sp. 998N23 previously isolated from *P. aphylla* (Table S2.5) (Lemaire & Muasya, unpublished), one of the few non-*Bradyrhizobium* associations identified for *A. saligna* in this study and also the only novel association for this species. Similarly, SW OTU3 is the dominant symbiont of *P. pinnata* in Grootbos soils, but nowhere else (Fig. 2.5). Blast results identified it as a *Mesorhizobium* previously isolated from the CCR legume *Otholobium bracteolatum* (Table S2.5) (Lemaire *et al.*, 2015). *Psoralea* spp. have been previously reported to share symbionts with this legume (Lemaire *et al.*, 2015). For both species, growth performances and BNF efficiencies were low in Grootbos soils. Intuitively, one would expect these non-dominant rhizobial associations to be responsible for the poor performances, however, *P. pinnata* has been shown to nodulate effectively with a wide range of *Mesorhizobium* strains (Kanu & Dakora, 2012; Lemaire *et al.*, 2015). On the other hand, there are few reports of *A. saligna* associating with *Mesorhizobium* strains (Boukhatem, Domergue, Bekki, *et al.*, 2012; Crisóstomo *et al.*, 2013), and it is plausible that these *Mesorhizobium* strains were ineffective partners/cheater strains, thereby hindering seedling performances. Nonetheless, *A. saligna* seedlings also associated with high relative abundances of SW OTU1 in Grootbos soils and, although the addition of Australian inoculum did improve *A. saligna*'s BNF (δ^{15}N; Fig. 2.1), this only translated into a marginal improvement in growth performance. Therefore, while these poor performances for both legume species could be attributed to ineffective novel associations, it is likely that the edaphic conditions of Grootbos soils may have played a greater role.

Overall, differences in soils, rather than inoculum addition, largely explained differences in the growth performance and BNF of both legumes. Similar distinctions in legume community composition as a result of edaphic characteristics have previously been found for CCR legumes (Dludlu *et al.*, 2018b), and these results may reflect variation in physiochemical and biotic properties between our soil collection sites. For example, as soil nitrogen assimilation is more energy efficient than BNF, soils with higher nutrient levels often lead to lower levels of BNF (higher δ^{15}N values; Fig. 2.1) (Heath & Tiffin, 2007). This was the case for *A. saligna* in *Psoralea*-conditioned soils where seedlings had significantly positive δ^{15}N values which were often higher than in the other soils (Fig. 2.1). Nonetheless, these seedlings also produced large numbers of nodules (Fig. 2.1) with a high overall dry weight

(Fig. S2.1). Together, this demonstrates that while these *A. saligna* seedlings may have been fixing atmospheric nitrogen, they were not necessarily wholly reliant on BNF to meet their nitrogen requirements since nitrogen could be assimilated directly from the soil. Increased nutrient levels are expected for this soil type since soils beneath well-established legume stands are often enriched for nitrogen (Chimphango, Potgieter & Cramer, 2015; Stirton *et al.*, 2015; Yelenik *et al.*, 2004). However, both species performed poorly in Grootbos, Kogelberg and Vergelegen soils and performed best in Rustenberg and *Psoralea*-conditioned soils, regardless of inoculum application (in most cases) or associations with preferred rhizobia (Fig. 2.1 and Fig. 2.5). These poor performances were prevelant even in cases where BNF was sufficient. Therefore, they are likely due to soil-specific biotic interactions, either through rhizobial cheater strains (Porter, Stanton & Rice, 2011) or higher pathogen loads (Thrall *et al.*, 2007), or differences in soil edaphic conditions, such as water-holding capacity (*A. saligna* – Bar (Kutiel), Cohen & Shoshany, 2004; *P. pinnata* – Bello *et al.*, 2017). Aside from the novel association for *A. saligna* in Grootbos soils, *P. pinnata* seedlings grown in Vergelegen soils also associated with different genera of rhizobia between the two inoculum treatments. In the absence of Australian inoculum, seedlings associated with the dominant *Mesorhizobium* SW OTU2. However, associations predominantly involved the *Bradyrhizobium* SW OTU1 when Australian inoculum was added, the only case of a novel association for *P. pinnata* in this study. The latter association, coupled with poor growth performances, would intuitively signal that the this symbiosis between *P. pinnata* and novel *Bradyrhizobium* strains is ineffective and potentially bordering on parasitism (Denison & Kiers, 2004; Rodríguez-Echeverría *et al.*, 2012). Yet, the relatively poor performance of *P. pinnata* in Vergelegen soil was similar under both inoculum application treatments, i.e. when associating with their preferred as well as novel rhizobia. *Acacia saligna* also performed poorly in these soils, even when associating with *Bradyrhizobium* in both inoculum treatments. This generally poorer performance of both legumes in Vergelegen soils, even when in the presence of their familiar rhizobia, further supports the notion that site-specific conditions, in terms of pathogen load/soil abiotic conditions, may more strongly impact the performance of these two legumes, rather than the availability of their preferred, and somewhat cosmopolitan, rhizobia.

I acknowledge that there is a possibility that the lack of a significant inoculation effect, particularly for *A. saligna*, may have resulted from extensive cross-contamination in the glasshouse. However, several considerations suggest that cross-contamination is an unlikely

explanation for the dominance of the same *Bradyrhizobium* strains in acacia root nodules collected from seedlings grown in inoculated and uninoculated soils. Firstly, stringent measures to minimize cross-contamination were put in place during the soil collection stages as well as the watering, randomizing and inoculation applications that took place during the glasshouse experiment (see Methods pages 18-20). Furthermore, Keet *et al.* (2017) sequenced root nodule communities from 19 different Australian acacia species (including *A. saligna*) sampled across a wide geographic range (up to 900 km apart) in South Africa. They used the same DNA barcode as I did in the current study. These authors found that all acacias shared a few, but highly abundant, *Bradyrhizobium* OTUs. The most dominant OTU identified by Keet *et al.* (2017), JHK OTU1, comprising 49% of their 98,000 DNA sequence reads, is also the most dominant *Bradyrhizobium* strain in this study, SW OTU1 (Fig. 2.5). More recently, Le Roux *et al.* (2018) characterized rhizobial communities in acacia-invaded and uninvaded CCR soils. They found that acacia invasion (by six different acacia species, including *A. saligna*) affects both the diversity and structure of soil rhizobial communities by lowering diversity and homogenizing community structure in favour of *Bradyrhizobium* strains in invaded compared to uninvaded soils. Again, one of the most dominant *Bradyrhizobium* OTUs identified from acacia-invaded soils in this study, JLR OTU1, comprising 9.85% of the 99,600 DNA sequence reads, corresponds to Keet *et al.* (2017) JHK OTU1, and thus my SW OTU1. Additionally, both Rustenberg Winery and Vergelegen Wine Farm sites used in this study for soil collections were included for soil sampling by Le Roux *et al.* (2018). Also, relative abundances of *Bradyrhizobium* strains isolated from paired invaded and uninvaded areas for these two sites were not significantly different (Le Roux *et al.*, 2018). Together with the known history of cointroduction of Australian *Bradyrhizobium* and acacias to South Africa (Ndlovu *et al.*, 2013; Warrington *et al.*, 2019), these findings strongly suggest that the most parsimonious explanation for the dominance of the same Australian bradyrhizobia in *A. saligna* nodules between treatments is that they are already established and widespread in South African soils.

The fact that exotic Australian *Bradyrhizobium* strains are established and widespread within CCR soils is troubling. My study adds to a growing body of literature suggesting that rhizobial mutualist availability is no longer a major limiting factor during acacia invasions (see Wandrag *et al.* (2020) and references therein). Additionally, there was one instance of a novel association for *A. saligna* in this study, although poor seedling performances appeared to be due to soil edaphic characteristics rather than this novel association. In fact, *A. saligna* has been

found to be a promiscuous host (Amrani, Noureddine, Bhatnagar, *et al.*, 2010; Boukhatem *et al.*, 2012; Crisóstomo *et al.*, 2013), yet several studies have also found that acacia host promiscuity does not necessarily relate to invasiveness (Keet *et al.*, 2017; Klock *et al.*, 2015). However, this is largly due to the many *Acacia* spp. being able to utilize (often co-introduced) *Bradyrhizobium* strains interchangeably (Keet *et al.*, 2017; Wandrag *et al.*, 2013; Warrington *et al.*, 2019). Therefore, cointroduction of effective rhizobial partners and host promiscuity are not mutually exclusive, but may act synergistically to enhance the colonization success and invasiveness of acacias. Taken together, the high abundances of Australian bradyrhizobia already present in pristine CCR soils suggest that this region is highly susceptible to future invasion by acacias currently not present in the country, i.e. invasion meltdown. Aside from the detrimental above-ground biodiversity impacts of acacia invasion, belowground biodiversity changes due to the presence of exotic bradyrhizobia also need to be considered. The CCR is home to a high diversity of rhizobia, such as *Burkholderia* and *Mesorhizobium* (Dludlu *et al.*, 2018a; Le Maitre *et al.*, 2011; Sprent *et al.*, 2017). While there were no notable negative impacts of exotic bradyrhizobia presence on the *Mesorhizobium* strains found in this study, the same may not be true for other rhizobial genera, or the legume hosts dependent on these strains. This is especially true considering the homogenization of acacia invaded soils in favour of *Bradyrhizobium* (Le Roux *et al.*, 2018; Slabbert *et al.*, 2014; Weir *et al.*, 2004) and the poor passive recovery of native vegetation following acacia clearing (Reinecke *et al.*, 2008). Future studies should investigate a variety of native CCR legumes nodulated by different native rhizobial strains in order to gain a clearer understanding of the overall impacts of exotic *Bradyrhziobium* on native legume-rhizobium symbioses.

Tables and Figures

***Table 2.1**: Results of generalized linear mixed models comparing the different growth performance and BNF measurements between different site and inoculum addition treatment combinations for* Acacia saligna *and* Psoralea pinnata.

		Acacia saligna				***Psoralea pinnata***			
		Num Df	Den Df	F-value	p-value	Num Df	Den Df	F-value	p-value
Seedling total dry	(Intercept)	1	76	8.1291	0.0056	1	88	7.0968	0.0092
	Inoculum	1	76	3.0698	ns	1	88	1.7205	ns
	Site	4	76	25.858	<0.0001	4	88	69.897	<0.0001
	Inoculum:site	4	76	3.1781	0.0181	4	88	0.7444	ns

root:shoot	(Intercept)	1	76	25.9591	<0.0001	1	88	37.169	<0.0001
	Inoculum	1	76	3.8723	0.0527	1	88	0.0219	ns
	Site	4	76	8.4474	<0.0001	4	88	6.6892	0.0001
	Inoculum:site	4	76	1.8652	ns	4	88	0.38	ns
Number of nodules	(Intercept)	1	76	30.766	<0.0001	1	88	9.0779	0.0024
	Inoculum	1	76	5.7156	0.0193	1	88	0.7955	ns
	Site	4	76	15.3216	<0.0001	4	88	50.931	<0.0001
	Inoculum:site	4	76	1.1736	ns	4	88	2.4495	0.052
$\delta^{15}N$	(Intercept)	1	76	35.317	<0.0001	1	88	92.794	<0.0001
	Inoculum	1	76	0.2922	ns	1	88	0.5345	ns
	Site	4	76	9.5352	<0.0001	4	88	11.291	<0.0001
	Inoculum:site	4	76	2.5067	0.0489	4	88	0.2392	ns

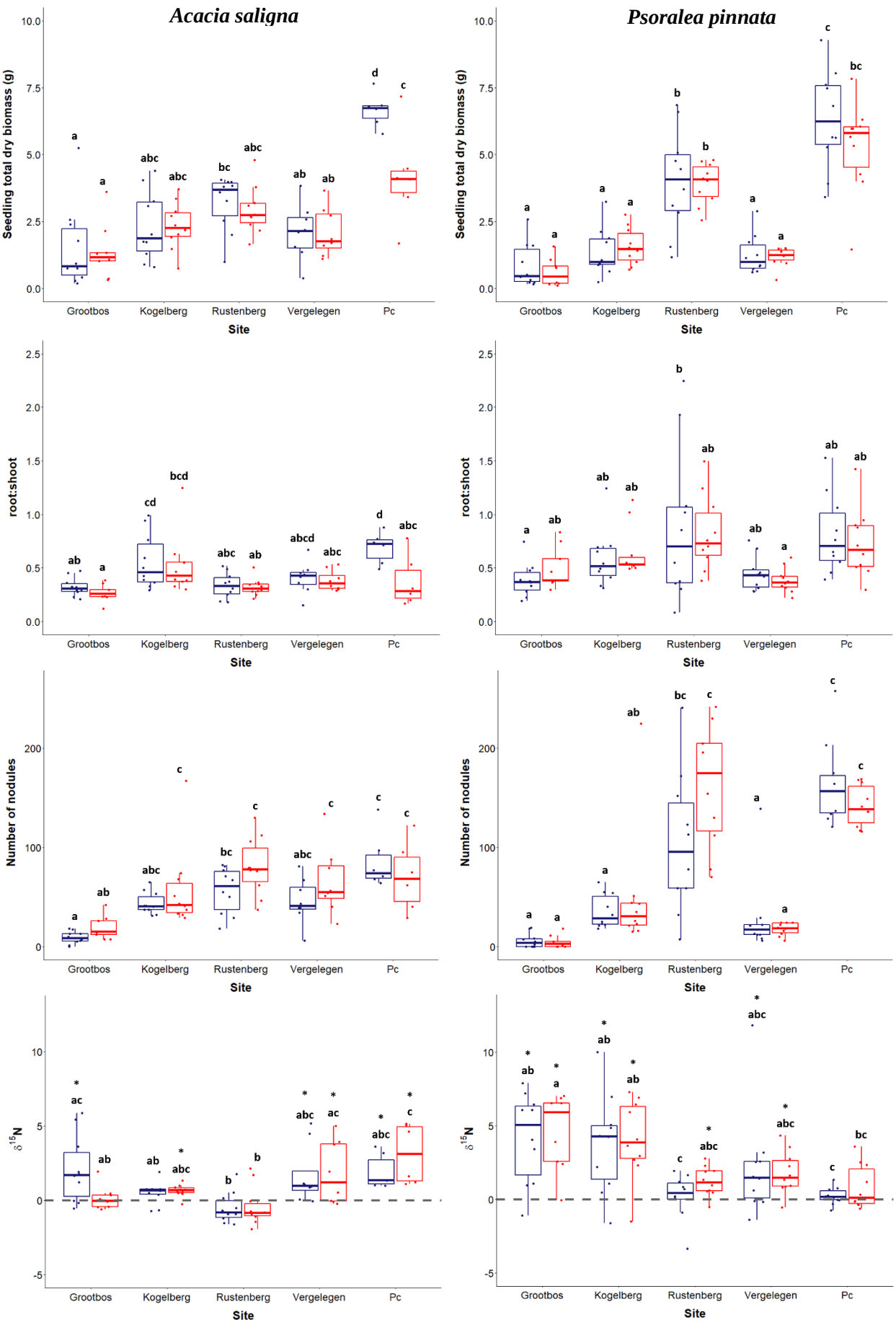


***Figure 2.1**: Growth performance (seedling total dry biomass and root:shoot ratio) and BNF (number of nodules and $\delta^{15}N$) measurements for* Acacia saligna *(left) and* Psoralea pinnata *(right) for each site (Grootbos, Kogelberg, Rustenberg, Vergelegen and* Psoralea*-conditioned (Pc) soils) by inoculum treatment (red – Australian inoculum added; blue – no inoculum added) combination. The broken horizontal line in the $\delta^{15}N$ graphs indicate where $\delta^{15}N = 0$. The * indicates which $\delta^{15}N$ values for each site by inoculum treatment combination is significantly different to zero.*

***Table 2.2**: Results of anova of generalized linear mixed models for* Acacia saligna *(type I sum of squares) and* Psoralea pinnata *(type III sum of squares) for the relationship between seedling root biomass and nodule number.*

	Acacia saligna				***Psoralea pinnata***		
	Num Df	Den Df	F-value	p-value	χ^2	Df	P-value
(Intercept)	1	76	39.4273	<0.0001	37.4066	1	<0.0001
Seedling root biomass	1	76	7.411	0.008	1.5263	1	ns
Inoculum	1	76	9.2869	0.0032	4.836	1	0.0279
Seedling root biomass:Inoculum	1	76	0.2883	ns	12.5765	1	0.0004

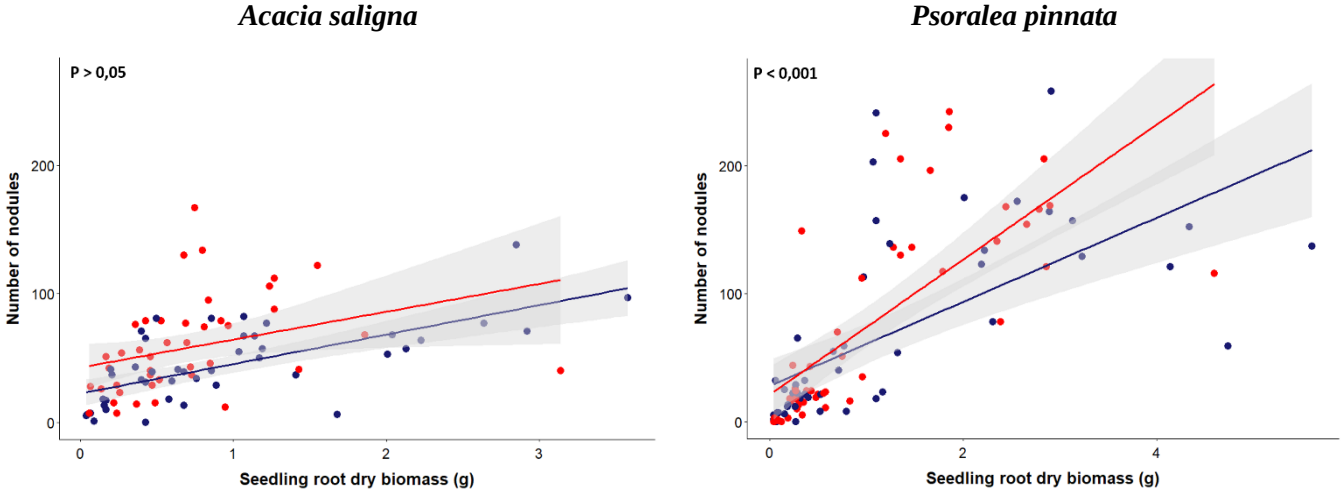


***Figure 2.2**: The encounter rate of rhizobia as indicated by the influence of root dry biomass on nodule formation in* Acacia saligna *(left) and* Psoralea pinnata *(right) seedlings for each inoculum treatment (red – Australian inoculum added; blue – no inoculum added). A significant interaction between the seedling root dry biomass and the Australian inoculum addition occurred for* P. pinnata*, but not for* A. saligna*.*

***Table 2.3**: Results of anova's of fixed effects from generalized linear mixed models investigating the influence of inoculation on the relationship between symbiotic interaction intensity (number of nodules) and growth performance and BNF for* Acacia saligna *and* Psoralea pinnata *seedlings.*

		Acacia saligna				*Psoralea pinnata*			
		Num Df	Den Df	F-value	p-value	Num Df	Den Df	F-value	p-value
Seedling total dry biomass	(Intercept)	1	76	33.185	<0.0001	1	88	7.7262	0.0067
	Nodule number	1	76	8.2746	0.0052	1	88	1.0542	ns
	Inoculum	1	76	6.7631	0.0112	1	88	2.7011	ns
	Nodule number: Inoculum	1	76	5.7103	0.0193	1	88	0.9551	ns
root:shoot	(Intercept)	1	76	95.106	<0.0001	1	88	149.82	<0.0001
	Nodule number	1	76	5.7249	0.0192	1	88	9.7959	0.0024
	Inoculum	1	76	7.2779	0.0086	1	88	0.2822	ns
	Nodule number: Inoculum	1	76	4.3684	0.04	1	88	0.3393	ns
$\delta^{15}N$	(Intercept)	1	76	3.1734	ns	1	88	7.4254	0.0078
	Nodule number	1	76	9.9139	0.0023	1	88	0.2492	ns
	Inoculum	1	76	0.0386	ns	1	88	0.3297	ns
	Nodule number: Inoculum	1	76	3.6837	0.0587	1	88	1.3175	ns

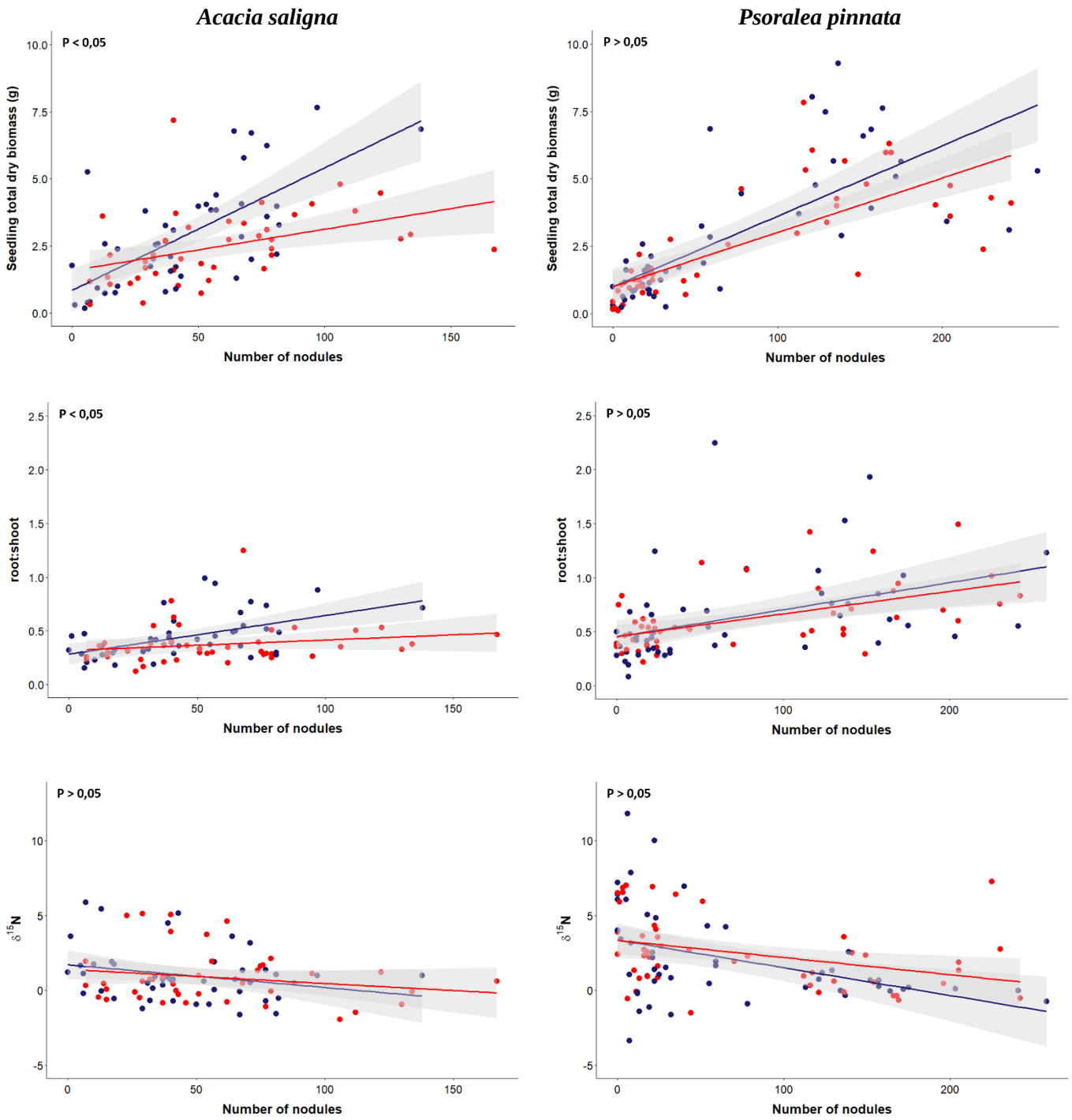


***Figure 2.3**: The contribution of nodules to the growth performance and BNF of* Acacia saligna *(left) and* Psoralea pinnata *(right) seedlings for all sites combined and the influence of inoculum treatment (red – Australian inoculum added; blue – no inoculum added) on each. There is only a significant interaction between nodule number and Australian inoculum addition for seedling total dry biomass and root:shoot ratios for* A. saligna*, but not for* $\delta^{15}N$ *of* A. saligna *or any of the measures for* P. pinnata.

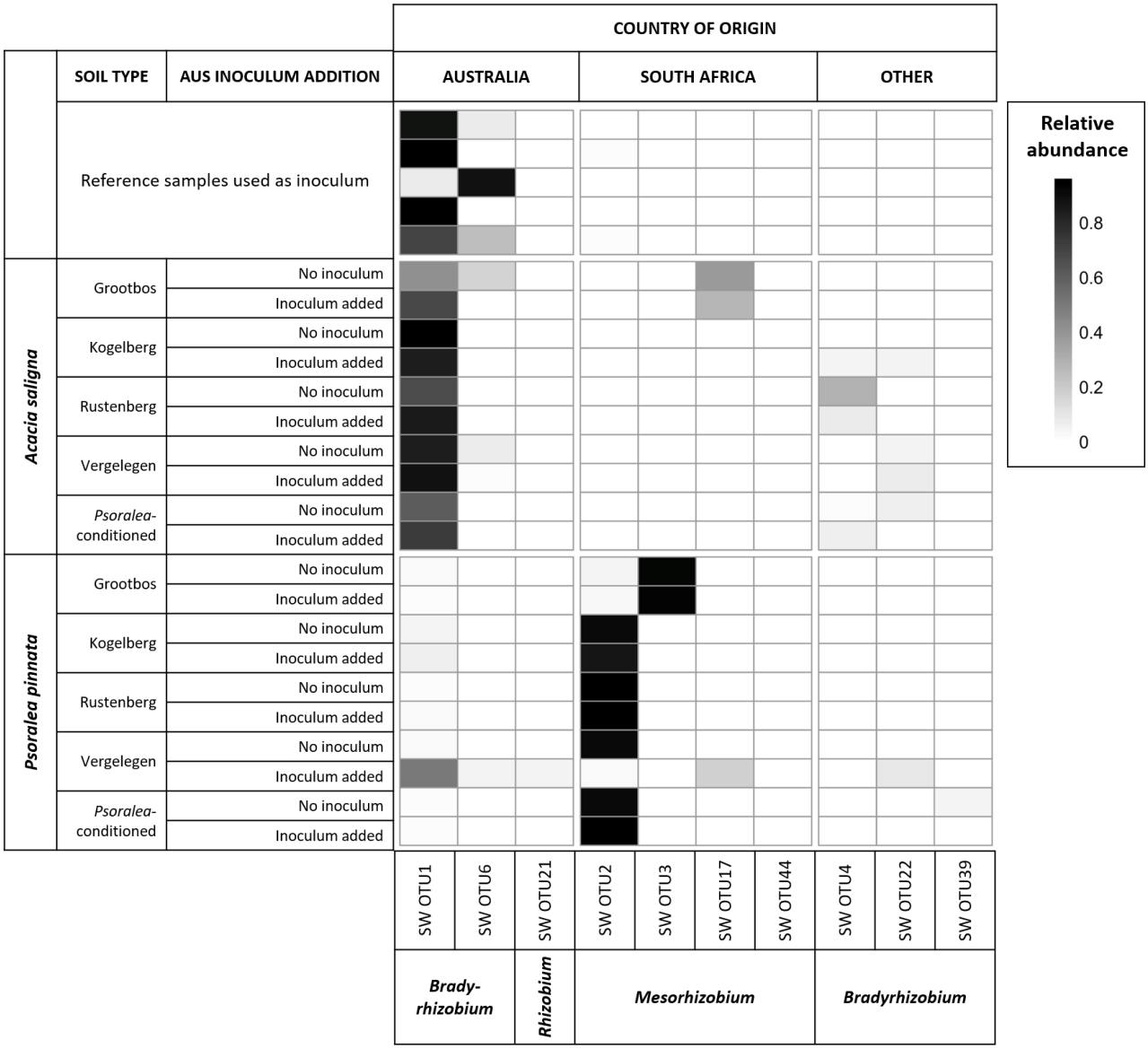


***Figure 2.4**: Heatmap based on the relative abundances of the 10 rhizobial OTUs identified in this research chapter. Darker shades represent higher relative abundances. OTUs are arranged according to country of origin (top x-axis) based on blast results and phylogenetic analyses (see Fig. 2.5). Y-axis labels show the reference samples used as inoculum as well as the 20 species x soil x inoculum addition treatment combinations. OTU labels and genus identity based on blast results are given on the bottom x-axis.*

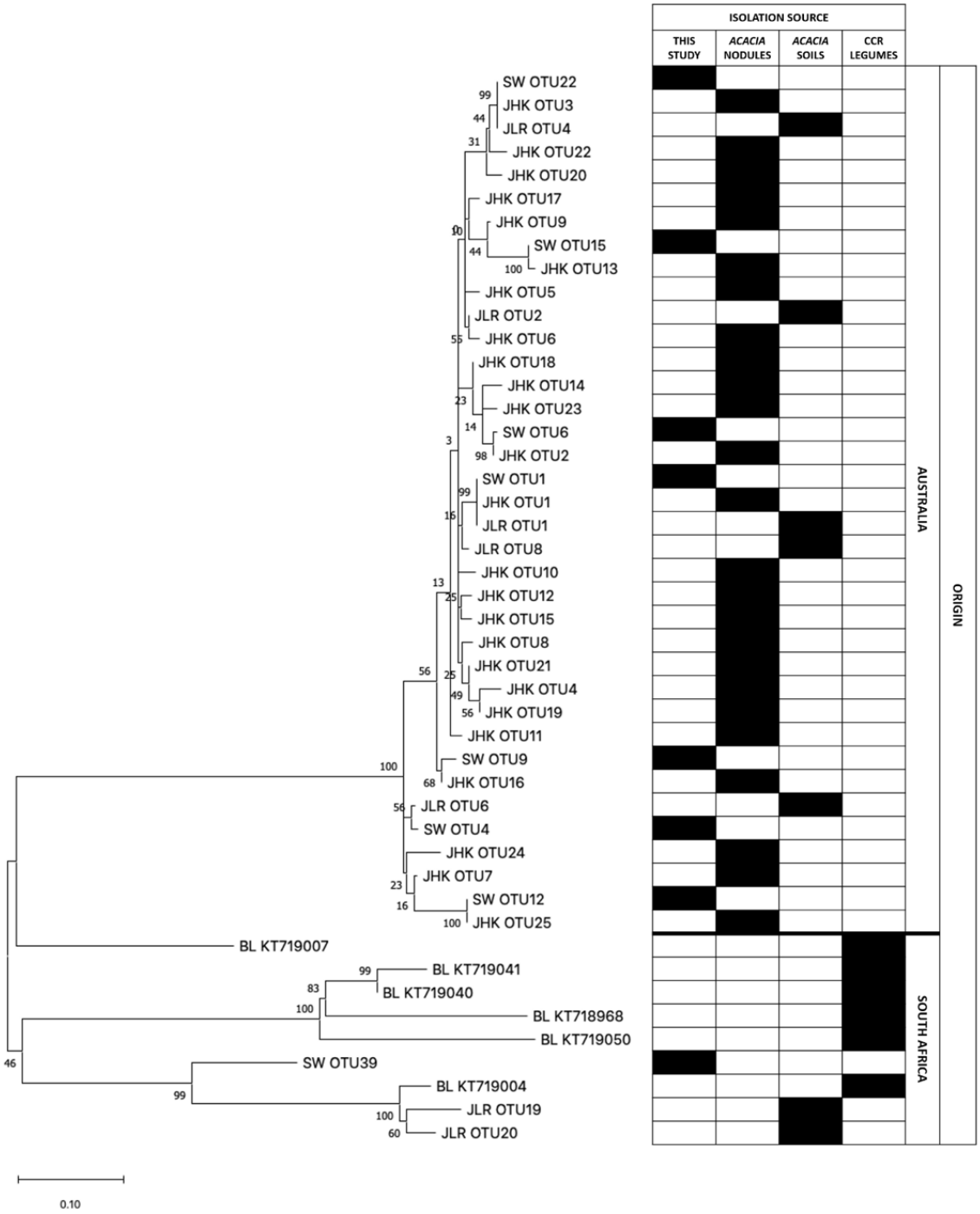


***Figure 2.5**: Maximum Likelihood phylogenetic tree showing the relationships between* nodC *sequences of* Bradyrhziobium *strains for this study (SW OTU) as well as those sequences previously isolated from acacia soils (JLR OTUs), acacia nodules (JHK OTUs) and CCR legumes (BL accessions) as indicated by the shaded blocks in the corresponding table. Tree is drawn to scale with branch length measured in the number of substitutions per site. Nodal support is given as bootstrap values.*

Supplementary Materials

***Table S2.1**: Co-ordinates of soil collection sites.*

Soil	Site	Co-ordinates
Grootbos	Grootbos Private Nature Reserve	-34.53579, 19.41586
Kogelberg	Kogelberg Nature Reserve	-34.16546, 18.94859
Rustenberg	Rustenberg Winery	-33.87257, 18.90123
Vergelegen	Vergelegen Wine Farm	-34.05489, 18.91027
Psoralea-conditioned	Prawn river lagoon Kogelberg Nature Reserve Vergelegen Wine Farm	-34.40743, 19.32842 -34.17037, 18.95083 -34.05532, 18.94745

***Tables S2.2**: Anova results of fixed effects for generalized linear mixed models comparing between different site and inoculum addition treatment combinations for* Acacia saligna *and* Psoralea pinnata *for the remaining growth performance and BNF measures.*

		Acacia saligna				***Psoralea pinnata***			
		Num Df	Den Df	F-value	p-value	Num Df	Den Df	F-value	p-value
Seedling height	(Intercept)	1	76	16.8674	0.0001	1	88	122.4519	<0.0001
	Inoculum	1	76	0.2968	ns	1	88	1.6482	ns
	Site	4	76	12.6957	<0.0001	4	88	61.3753	<0.0001
	Inoculum:site	4	76	0.0621	ns	4	88	1.2405	ns
Seedling shoot dry biomass	(Intercept)	1	76	9.6358	0.0027	1	88	12.3382	0.0007
	Inoculum	1	76	1.0057	ns	1	88	1.3136	ns
	Site	4	76	26.5447	<0.0001	4	88	107.2272	<0.0001
	Inoculum:site	4	76	1.579	ns	4	88	0.9722	ns
Seedling root dry biomass	(Intercept)	1	76	6.4086	0.0134	1	88	4.2243	0.0428
	Inoculum	1	76	6.0564	0.0161	1	88	1.2528	ns
	Site	4	76	19.4725	<0.0001	4	88	30.2051	<0.0001
	Inoculum:site	4	76	4.97	0.0013	4	88	0.4352	ns
Nodule total dry biomass	(Intercept)	1	76	6.5747	0.0123	1	88	3.3129	ns
	Inoculum	1	76	0.0646	ns	1	88	0.0771	ns
	Site	4	76	16.2545	<0.0001	4	88	60.3405	<0.0001
	Inoculum:site	4	76	1.7074	ns	4	88	0.2674	ns

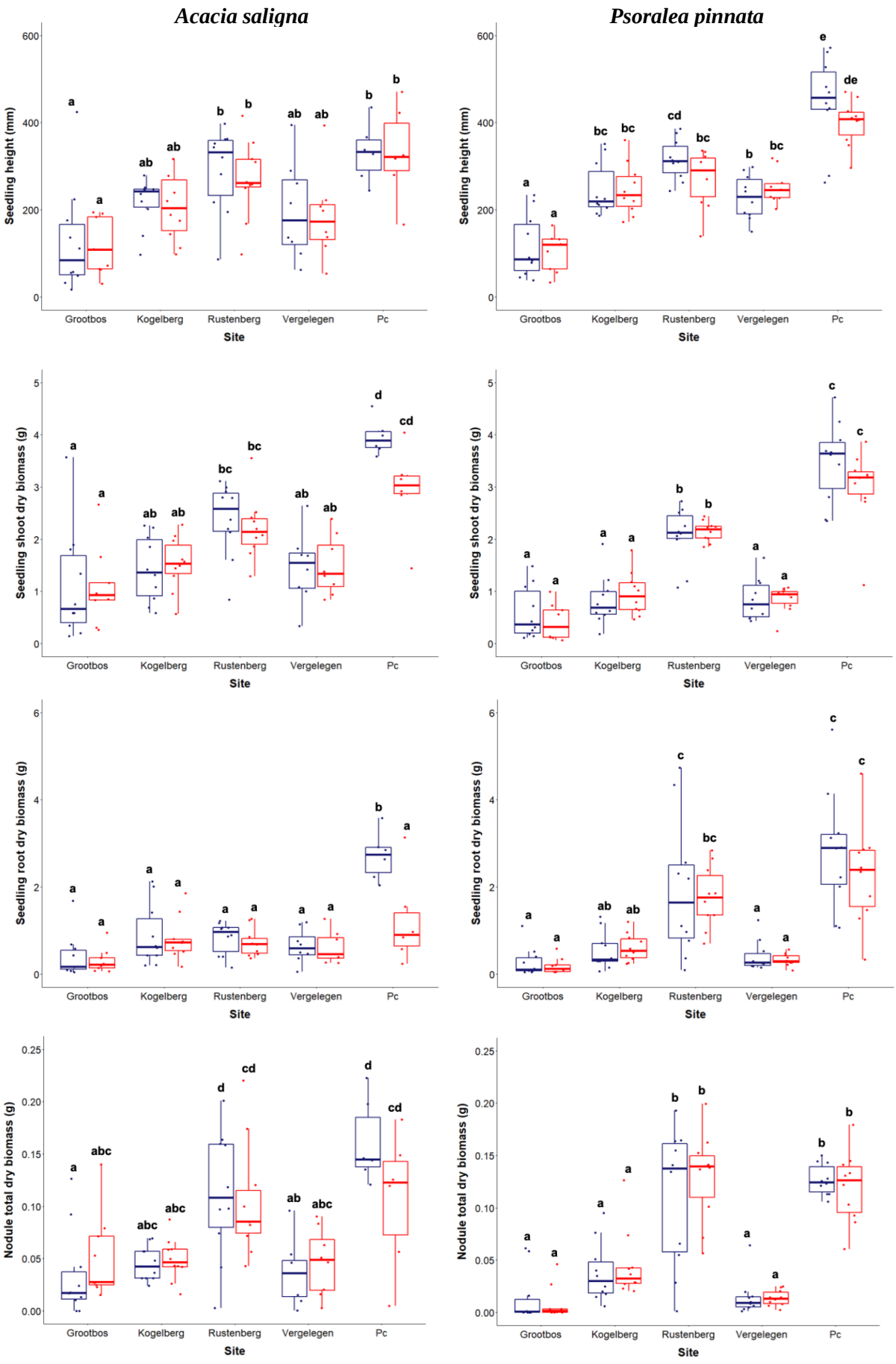


***Figure S2.1**: The remaining growth performance (seedling height, and seedling shoot and root dry biomass) and BNF (nodule total dry biomass) measurements for* Acacia saligna *(left) and* Psoralea pinnata *(right) for each site (Grootbos, Kogelberg, Rustenberg, Vergelegen and* Psoralea*-conditioned (Pc) soils) by inoculum treatment (red – Australian inoculum added; blue – no inoculum added) combination.*

***Table S2.3**: Anova results of fixed effects from generalized linear mixed models investigating the influence of inoculation on the relationship between symbiotic interaction intensity (number of nodules) and the remaining growth performance and BNF measurements for* Acacia saligna *and* Psoralea pinnata *seedlings.*

		Acacia saligna				***Psoralea pinnata***			
		Num Df	Den Df	F-value	p-value	Num Df	Den Df	F-value	p-value
Seedling height	(Intercept)	1	76	67.226	<0.0001	1	88	25.848	<0.0001
	Nodule number	1	76	3.0741	ns	1	88	0.1559	ns
	Inoculum	1	76	1.4066	ns	1	88	1.4731	ns
	Nodule number: Inoculum	1	76	0.8515	ns	1	88	0.4717	ns
Seedling shoot dry biomass	(Intercept)	1	76	32.703	<0.0001	1	88	9.1457	0.0033
	Nodule number	1	76	6.3934	0.0135	1	88	1.6004	ns
	Inoculum	1	76	3.2294	ns	1	88	1.6723	ns
	Nodule number: Inoculum	1	76	2.6596	ns	1	88	1.1161	ns
Nodule total dry biomass	(Intercept)	1	76	30.81	<0.0001	1	88	37.248	<0.0001
	Nodule number	1	76	13.814	0.0004	1	88	94.039	<0.0001
	Inoculum	1	76	1.8524	ns	1	88	0.5041	ns
	Nodule number: Inoculum	1	76	2.4321	ns	1	88	1.3954	ns

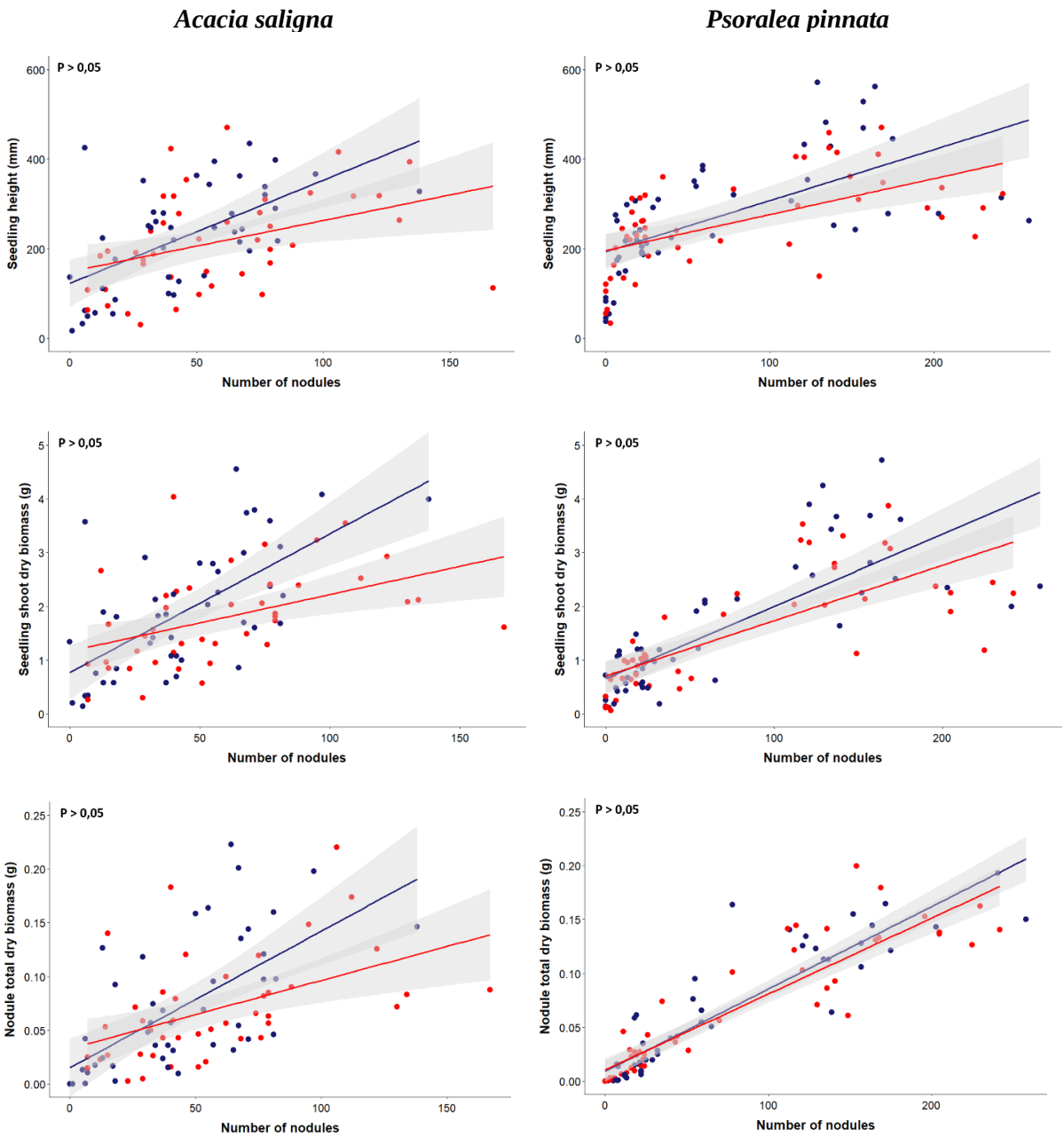


Figure S2.2: *The contribution of nodules to the remaining growth performance (seedling height and shoot dry biomass) and BNF (nodule total dry biomass) of* Acacia saligna *(left) and* Psoralea pinnata *(right) seedling for all soils combined and the influence of inoculum treatment (red – Australian inoculum added; blue – no inoculum added) on each. No significant interactions were found between nodule number and inoculum addition for any of the above measurements, shown by the 'P>0.05'.*

Table S2.4*: Marginal* R^2 *(fixed effects), conditional* R^2 *(overall model) and* R^2 *values of random effects showing the amount of variance explained by each based on the linear mixed models of nodule contribution to the growth performance and BNF measures of* Acacia saligna *and* Psoralea pinnata *seedlings.*

	Acacia saligna	***Psoralea pinnata***

	Marginal R^2	Conditional R^2	Random effects R^2	Marginal R^2	Conditional R^2	Random effects R^2
Seedling height	0.0661	0.3386	0.2725	0.0053	0.7701	0.7648
Seedling shoot dry biomass	0.1015	0.5819	0.4804	0.0152	0.8315	0.8163
Seedling total dry biomass	0.1665	0.5689	0.4024	0.019	0.7595	0.7405
root:shoot	0.1835	0.3657	0.1822	0.1431	0.2099	0.0668
Nodule total dry biomass	0.1953	0.4608	0.2655	0.6143	0.7966	0.1823
δ^{15}N	0.1286	0.532	0.4034	0.0155	0.4112	0.3957

***Table S2.5**: Blast results of the 13 OTUs identified for both research chapters of this thesis.*

OTU	Accession	% Identity	Genus	Species	Strain	Host plant	Origin	Reference	Applicable chapter
SW OTU1	KX289800	100	*Bradyrhizobium*	sp.	CPI240	*Acacia* spp.	AUS	Barrett et al., 2016	2 and 3
SW OTU2	KT719028	98.72	*Mesorhizobium*	sp.	969N9	*Psoralea fleta*	RSA	Lemaire & Muasya, unpublished	2 and 3
SW OTU3	KR154628	98.4	*Mesorhizobium*	sp.	OD42	*Otholobium bracteolatum*	RSA	Lemaire et al., 2015	2
SW OTU4	MG588292	98.99	*Bradyrhizobium*	*japonicum*	de_042-1	*Acacia dealbata*	USA	Urbina & Klock, unpublished	2 and 3
SW OTU6	KX289801	97.76	*Bradyrhizobium*	sp.	CPI241	*Acacia* spp.	AUS	Barrett et al., 2016	2 and 3

SW OTU9	KX289800	97.44	*Bradyrhizobium*	sp.	CPI240	*Acacia* spp.	AUS	Barrett et al., 2016	3
SW OTU12	MG588292	94.93	*Bradyrhizobium*	*japonicum*	de_042-1	*Acacia dealbata*	USA	Urbina & Klock, unpublished	3
SW OTU15	KX289800	97.44	*Bradyrhizobium*	sp.	CPI240	*Acacia* spp.	AUS	Barrett et al., 2016	3
SW OTU17	KT719013	97.44	*Mesorhizobium*	sp.	998N23	*Psoralea aphylla*	RSA	Lemaire & Muasya, unpublished	2
SW OTU21	KX289807	97.76	*Rhizobium*	sp.	CPI314	*Acacia* spp.	AUS	Barrett et al., 2016	2
SW OTU22	MG588285	100	*Bradyrhizobium*	*lupini*	de_01	*Acacia dealbata*	USA	Urbina & Klock, unpublished	2 and 3
SW OTU39	CP022219	87.16	*Bradyrhizobium*	*guangxiense*	CCBAU53363	*Arachis hypogaea*	CHI	Sui & Li, unpublished	2 and 3
SW OTU44	KT719013	95.85	*Mesorhizobium*	sp.	998N23	*Psoralea aphylla*	RSA	Lemaire & Muasya, unpublished	2 and 3

***Table S2.6**: Results of PERMANOVA analysis comparing the distance matrix of the 10 SW OTUs' relative abundances between species identity and inoculum addition treatments.*

	Df	**Sum of Squares**	***R²***	**Pseudo F-value**	**p-value**
Inoculum	1	0.0566	0.0106	0.4052	ns
Host species	1	2.999	0.5608	21.4853	0.001
Inoculum:Host species	1	0.0586	0.0110	0.4201	ns
Residual	16	2.2333	0.4176		

Total	19	5.3475	1

***Table S2.7**: % contribution of each SW OTU to the dissimiliarity of nodule rhizobial community composition between* Acacia saligna *and* Psoralea pinnata*. SW OTUs are ordered according to their % contribution.*

OTU	**% Contribution to dissimilarity**
SW OTU1	35.35
SW OTU2	34.01
SW OTU3	9.91
SW OTU17	3.96
SW OTU4	2.59
SW OTU6	1.60
SW OTU22	1.57
SW OTU21	0.30
SW OTU39	0.26
SW OTU44	0.02

Chapter 3

The role of invasion-associated alterations to soil abiotic and biotic conditions in facilitating competitiveness of an invasive legume.

Abstract

Positive-feedbacks, whereby organisms alter environmental conditions in ways that benefit their own performance, often allow invasive plants to outcompete natives. Invasive Australian acacias cause substantial biodiversity impacts in South Africa's Core Cape Subregion via positive-feedbacks driven by high growth rates and leaf litter input and their association with nitrogen-fixing rhizobia. However, while these attributes may act in synergy to create positive feedbacks, it is unclear whether acacia-induced (a)biotic changes (i.e. leaf litter input) or their association with different types of rhizobia (i.e. nitrogen fixation via co-introduced or novel rhizobia), or both, are more important in driving the invasive performance of acacias. Here I aim to determine i) whether familiar (i.e. Australian) vs novel (i.e. South African) rhizobia associations affect the competitive ability of *Acacia saligna* grown in competition with a native legume, *Psoralea pinnata* and ii) the relative contribution of acacia leaf litter and familiar rhizobial associations to the performance of *A. saligna* under competition. I grew *A. saligna* and *P. pinnata* alone and together in pots containing *Psoralea*-conditioned soils. Seedlings grown alone were only subjected to Australian bradyrhizobia inoculum treatments, while seedlings grown in mixture received a combination of inoculum and acacia-topsoil (i.e. soil conditioned by acacia leaf litter) in a fully factorial design. Relative Competition Indices (RCI) revealed that *A. saligna* and *P. pinnata* had similar competitive abilities, regardless of inoculum treatment. I found that neither the addition of Australian inoculum nor topsoil facilitated the performance of *A. saligna*. Furthermore, these treatments also did not hinder *P. pinnata*'s performance relative to *A. saligna*. Next generation sequencing (NGS) data revealed that each species associated with their familiar rhizobia (i.e. *A. saligna* associated with Australian bradyrhizobia while *P. pinnata* associated with CCR *Mesorhizobium*) regardless of inoculum application. δ^{15}N values revealed that *A. saligna* and *P. pinnata* were primarily utilizing soil nitrogen and atmospheric nitrogen, respectively. This apparent differentiation in the use of nitrogen and rhizobial niches may explain the absence of strong asymmetric competitive outcomes between these two species and account for the ability of *P. pinnata* to persist under Acacia invasion, highlighting its potential in active restoration projects. It also suggests that

Australian bradyrhizobia are prolific within CCR soils and, therefore, may no longer be a barrier to acacia establishment.

Introduction

Establishment success by introduced non-native plants is often linked to their high competitive ability for essential resources, such as light, water and nutrients (Morris *et al.*, 2011). Such competition is tightly linked to plant functional traits such as fast growth rates (Witkowski, 1991), increased seed set (Gibson, Richardson, Marchante, *et al.*, 2011), pathogen resistance, and nutrient acquisition through increased root biomass and/or microbial mutualisms (mycorrhiza or nitrogen-fixing bacteria) (Funk & Vitousek, 2007), etc. These traits often generate positive-feedback mechanisms (Gaertner *et al.*, 2014) which may vary between species and regions, but always tend to increase the invader's abundance while simultaneously decreasing that of co-occuring native species. Positive feedbacks can be complex, e.g. invasive cheatgrass (*Bromus tectorum*) in the Great Basin of the western United States causes significant changes in soil microbial communities (Belnap & Phillips, 2001) and function (nitrogen cycling; (Rimer & Evans, 2006) that increases fire fuel loads. All these changes have resulted in more frequent and intense fires that further benefitted cheatgrass through the competitive release from fire-intolerant native species (Chambers, Roundy, Blank, *et al.*, 2007).

In the case of invasive legumes, positive feedbacks are also multifaceted (Le Roux *et al.*, 2017). One of the dominant functional traits driving these positive feedbacks is the ability of most legumes to associate with nitrogen-fixing bacteria (rhizobia) that increases plant nutrient acquisition and growth rates. For example, Lau & Suwa (2016) found that invasive *Vicia villosa* experienced growth increases of up to 125% when in the presence of effective rhizobial partners compared to when these rhizobia were absent. In fact, rhizobium symbioses are thought to often drive plant-plant competitive interactions (Keller, 2014) as well as the homogenization of the soil biotic conditions in favor of the dominant legume's preferred rhizobial strains (Hortal, Lozano, Bastida, *et al.*, 2017). Under legume invasion, changes in soil biotic communities often occur in concert with changes in soil abiotic conditions through increased nitrogen and carbon deposition from roots and/or increased invader leaf litter input. This multifaceted feedback may increase the severity and the rate of impact accrual on co-occurring native species (Le Roux *et al.*, 2017), particularly in nutrient-poor environments

where co-occuring native plants are adapted to low nutrient conditions and thus unable to persist under soil conditions generated by invasive legumes (Yelenik *et al.*, 2004).

Australian acacias (genus *Acacia* Mill.) are some of the most impactful invasive trees globally (Richardson & Rejmánek, 2011), especially in Mediterranean-type ecosystems (Gaertner *et al.*, 2009; Le Maitre *et al.*, 2011). It is thought that acacia invasion often leads to positive feedbacks via changes in vegetation structure and soil chemistry, driven by a number of functional traits (Morris *et al.*, 2011). Acacias generally grow taller than the co-occurring schlerophyllous shrubs characteristic of Mediterranean ecosystems (Witkowski, 1991). This causes increased shading and light competition as well as higher production of leaf litter which is slow to decompose. Acacias also release allelopathic chemicals from leaf litter, flowers and roots which can inhibit growth of co-occurring native plants (Lorenzo, Palomera-Pérez, Reigosa, *et al.*, 2011; Abd El-Gawad & El-Amier, 2015). Moreover, acacias nodulate profusely in their non-native ranges (Europe – Rodríguez-Echeverría *et al.*, 2009; Asia – Le Roux, Tentchev, Prin, *et al.*, 2009; southern Africa – Ndlovu *et al.*, 2013; Le Roux *et al.*, 2016; the Americas – Aronson, Ovalle & Avendaño, 1992; New Zealand - Weir *et al.*, 2004). Changes in soil nutrients under acacia invasion may also act to enhance the survival and proliferation of their preferred rhizobial strains (be these exotic or native bradyrhizobia) as the rhizospheric biotic community composition of acacias is dependent upon the nutritional status of surrounding soils (Kamutando *et al.*, 2017). The absence of preferred rhizobia has been found to limit the growth of some non-native acacias when grown in soils away from established acacia populations (Wandrag *et al.*, 2013). However, these limitations are probably rare as acacias and their preferred Australian bradyrhizobial symbionts often co-invade habitats (for review see Wandrag *et al.*, 2020). The positive feedback mechanisms generated by acacias are thus integrated with soil chemical changes and *Bradyrhizobium* cointroductions acting synergistically to increase the competitive abilities of acacias.

One region where acacia-associated positive-feedbacks have been particularly apparent is South Africa's Core Cape Subregion (CCR; formerly the Cape Floristic Region; Gaertner *et al.*, 2014). The CCR is a globally-renowned biodiversity hotspot and home to plant species well-adapted to the region's characteristically nutrient poor and seasonally arid soils (Manning & Goldblatt, 2012; Read & Mitchell, 1983). Species have adapted to these low nutrient conditions through mycorrhizal associations and the use of proteoid cluster roots (Lamont,

1982), but perhaps the most prominent is the association between legumes and rhizobia. The Fabaceae family is the second largest in the CCR with approximately 764 species (Manning & Goldblatt, 2012). CCR legumes associate with a diverse array of *Paraburkholderia* (formerly *Burkholderia*; *beta*-Proteobacteria), *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* (*alpha*-Proteobacteria) (Beukes *et al.*, 2013; Elliott *et al.*, 2007; Gerding, O'Hara, Bräu, *et al.*, 2012; Hassen *et al.*, 2012; Kanu & Dakora, 2012; Kock, 2004; Lemaire *et al.*, 2015; du Preez, 2019). Australian acacias are the most widespread invasive species in the CCR, posing a major threat to the region's unique biodiversity (Le Maitre *et al.*, 2011). They enrich CCR soils for nitrogen that promotes their own performance, to the detriment of native species (Yelenik *et al.*, 2004). Previous work has found many acacias to have been co-introduced into the CCR with a few co-evolved Australian bradyrhizobia (i.e. familiar associations; (Ndlovu *et al.*, 2013; Le Roux *et al.*, 2016; Warrington *et al.*, 2019) and it has been hypothesised that acacia-induced changes in abiotic soil conditions may also facilitate the survival of their preferred bradyrhizobia, while simultaneously jeopardizing the survival of native CCR rhizobial strains (Le Roux *et al.*, 2017). Moreover, most invasive acacias in the CCR share one or two Australian *Bradyrhizobium* strains (Keet *et al.*, 2017), suggesting that congeners may benefit from the enrichment of acacia-specific bradyrhizobia by already-invasive acacias. Therefore, impacts on native plants, particularly legumes, may be two-fold, in that not only are they not adapted to the altered soil abiotic conditions created by the acacia invasions, but the abundances of their preferred rhizobia may also decline if Australian rhizobia outcompete native strains for associations with CCR legumes. The latter may result in ineffective biological nitrogen fixation (BNF), further compromising native legume survival (e.g. see Rodríguez-Echeverría *et al.*, 2012).

Although positive feedbacks are multifaceted, the relative contribution of their individual components to the competitive performance of invasive species is not well understood, and certainly not for acacias. Therefore, this chapter aims to determine (i) whether the presence of exotic Australian *Bradyrhizobium* strains (i.e. familiar association) alters the competitive dynamics between an Australian acacia and a native legume (i.e. novel association) in favor of the former and (ii) the relative contribution of exotic *Bradyrhizobium* strains and acacia-induced abiotic soil changes (through leaf-litter input), individually and synergistically, in facilitating acacia performance under competition with a native legume. This was done by growing invasive *Acacia saligna* and native *Psoralea pinnata* seedlings in a two-part

competition experiment where they were exposed to a variety of competition treatments (grown alone and together), Australian *Bradyrhizobium* inoculum and *Acacia*-topsoil (i.e. the soil layer directly below the leaf litter layer) addition treatment combinations. I also used next-generation sequencing approaches to confirm whether familiar or novel rhizobial associations were formed by each legume. I hypothesized that the presence of exotic *Bradyrhizobium* strains would facilitate the competitive ability of *A. saligna* (i.e. via familiar associations) as shown by an increase in growth performance and BNF relative to *P. pinnata* when grown under competition. Additionally, I expected *Acacia* performance to improve under either increased availability of Australian bradyrhizobia or acacia topsoil, and that it should be best in the presence of both these factors acting in synergy. Finally, I expected that negative impacts on *P. pinnata* performance will increase in a similar manner, as shown by the concurrent reduction in performance across these treatments.

Methods

Study system

The same two species that were used in the glasshouse experiment of Chapter 2 were also used in this experiment. Briefly, *Acacia saligna* (Labill.) Wendl., commonly known as Port Jackson willow, is invasive in South Africa and have many devastating ecological impacts (Le Maitre *et al.*, 2011) through the generation of positive feedback mechanisms (Gaertner *et al.*, 2014; Yelenik *et al.*, 2004). *Psoralea pinnata* L., commonly known as fountain bush, is native to the CCR and is one of few native CCR legumes that co-occur with invasive acacias (personal observation). This overlap in distribution may be due to *A. saligna* and *P. pinnata* utilizing predominantly different rhizobial genera, i.e. *Bradyrhizobium* and *Mesorhizobium*, respectively. Considering this possibility, a comparison between *A. saligna* and *P. pinnata* under a competition scenario may provide interesting insights into the influence of rhizobia and host-plant specificity on plant-plant competitive interactions.

Soil collections, Australian inoculum preparation and glasshouse experimental setup

In order to mimic conditions when colonizing acacias invade an area already dominated by *P. pinnata*, soils were collected from beneath *P. pinnata* shrubs in the field during October 2018. Soils were collected from the same five *P. pinnata* shrubs at the same three sites (Prawn Lake in Hermanus, Kogelberg Nature Reserve and Vergelegen Wine Farm, see Table S3.1 for details) as the *Psoralea*-conditioned soils used in Chapter 2. The same soil collection protocols

were followed as in Chapter 2 with a few exceptions. A total of 40L of soil was collected from within a 1m radius of each *P. pinnata* shrub and bulked, and thoroughly mixed, to make up a total of 200L of soil. Soils were stored in two sterile 110L opaque plastic storage containers and all equiptment was sterilized with 70% ethanol between collections. At the end of the collection period, the bulked soil was sieved through a sterile 4mm mesh in order to remove any plant debris and rocks. Soils were then returned to storage containers and stored at room temperature for a period of 3 months before commencing with the glasshouse experiment.

A layer of drainage chips followed by 2L of the collected *Psoralea*-conditioned soils were placed into green plastic gardening pots (18cm diameter x 15.5cm height) which were each placed onto a water collecting saucer (20cm diameter). This was done for a total of 40 pots. Equipment used during this process was sterilized with 70% ethanol prior to placing soils into pots. All pots were then watered with tap water until water-saturated. Seeds of *A. saligna*, collected from invasive populations within the Western Cape, were obtained from the Agricultural Research Centre's Plant Protection Research Institute (ARC-PPRI) in Stellenbosch. *Psoralea pinnata* seeds, collected from populations across the Cape Peninsula, were supplied by Silverhill Seeds in Kenilworth, Cape Town. All seeds were surface-sterilized and scarified prior to planting according to protocols described in Chapter 2. Three seeds of each species were placed into each of the 40 pots. Seeds were germinated and the seedlings allowed to establish for a period of five weeks, after which all but one seedling per species were haphazardly removed from pots (i.e. each pot contained only one *A. saligna* seedling and one *P. pinnata* seedling). In a few pots, there was no germination of any seeds for either one of the two species or for both species. To make up for these losses, extra seedlings removed from pots with high germination success were transplanted into these pots, within the same treatment combinations.

As a means to ensure that rhizobial communities were still present within the soils post-storage, fresh soil was collected from each of the five *P. pinnata* shrubs and applied to the pots as a soil inoculum (van de Voorde *et al.*, 2012). These collections were conducted according to the same protocol as the initial collections, except only 2L of soil were collected per shrub. Six weeks post-planting, 0.2L of this fresh soil was added to all pots, taking care not to smother seedlings in the process. All equipment was sterilized with 70% ethanol prior to adding soil inocula. Australian inoculum and *A. saligna* topsoil (i.e. leaf litter-conditioned soil) were added

to the 40 pots in a fully factorial design (four treatment combinations – with/without Australian inoculum and with/without topsoil addition, with ten replicates each). One week after soil inoculum addition, an Australian rhizobium inoculum was applied (i.e. 7 weeks post-planting). The same inoculum preparation protocols were used as specified in Chapter 2. 5mL of inoculum were added to half of the pots (n=20) and a sterile Yeast Mannitol broth was added to the remaining half. Inoculum application was repeated four weeks later. *Acacia saligna* topsoil was collected from a well-established invasive population of *A. saligna* adjacent to Vrede Wine Farm in Stellenbosch (See Table S3.1 for details). The top layer of loose leaf material was moved aside and a total of 5L of soils were collected from directly beneath this layer and stored within a 10L sterile opaque plastic container. This top layer of soil was used instead of actual leaf litter as it best represented the soil chemical changes induced by leaf-litter breakdown. These soils were sieved through a sterile 4mm mesh to remove any large debri and then sterilized using a laboratory autoclave to ensure that novel microbes present at the collection site were not introduced to the pots receiving topsoil. Once the soils had cooled, 100mL of this topsoil was added to 20 of the pots with care taken not to smother seedlings in the process. This was done two weeks after the first batch of Australian inoculum was added to the seedlings and again every two weeks for the duration of the experiment so as to simulate conditions similar to the onset of positive feedbacks driven by acacia leaf litter inputs. Fresh acacia topsoil was collected from the same site and processed as above prior to each application.

During the initial five week germination period, and prior to Australian inoculum addition, all plants were watered with tap water using a watering can. Once inoculum had been applied, a more stringent watering system was put in place whereby each pot individually received between 100mL to 200mL of tap water every second day for the duration of the experiment. All plants received the same volume of water on any given watering day with the amount dependent on how dry the majority of saucers were at the time of watering. Plants were grown in a glasshouse exposed to ambient light and temperature conditions for a total of 17 weeks from day of planting (February 2019) to harvest (June 2019) of which five weeks were set aside for germination/seedling establishment and the remaining 12 weeks were under the various inoculum and acacia topsoil addition treatments. All pots were randomly placed within the glasshouse and randomized once a week so as the minimize any effects on growth that

microclimates within the glasshouse may have. Seedlings were harvested at the end of the 17-week period.

Data collection

To harvest seedlings with minimal damage to their root systems, pots were vigorously tapped to loosen the soil. The two seedlings and the pot were then inverted to remove the pot. Soil was carefully shaken off the roots so that negligible numbers of nodules were lost during the process. All excess soil was rinsed off by dunking the root systems of the two seedlings into a bucket of tap water and the roots were dabbed dry using tissue paper. As the roots of the two seedlings had grown intertwined, special care was taken to disentangle them with minimal breakage to the root systems. Any nodules that had detached during this process were easily identified as belonging to one of the two legume species as nodule morphology was linked to host species identity (round determinate nodules were found on *P. pinnata*; also see Kanu & Dakora, 2012) and irregular indeterminate nodules were found on *A. saligna* (also see Sprent *et al.*, 2017). Nodules of each species were stored separately in tubes containing silica crystals. All growth performance and BNF measures were taken following the protocols outlined in Chapter 2. These measurements included seedling height, seedling shoot dry biomass and seedling root dry biomass as well as total nodule number and δ^{15}N, for growth performances and BNF, respectively. This was done for each seedling per pot.

In addition to the fully factorial competition experiment, *A. saligna* and *P. pinnata* seedlings were also grown in separate pots and exposed to the Australian inoculum addition treatments only (7 to 10 replicate pots per treatment). These pots were filled with the same *Psoralea*-conditioned soils as for those seedlings grown in mixture and were also treated in the same manner regarding soil inoculum application, Australian inoculum application, watering and randomization. All growth performance and BNF measures were also recorded for these seedlings at harvest. This allowed me to make comparisons of seedling performance between those seedlings grown alone in separate pots (Chapter 2) and those grown in mixture in a single pot (this chapter).

Statistical analyses of growth performance and BNF measurements

All statistical analyses were conducted in the R statistical environment (v3.4.4 R Core Development Team).

Growth performance (seedling height, seedling shoot dry biomass, seedling root dry biomass) and BNF (nodule number and δ^{15}N) measures for seedlings of both species grown alone in *Psoralea*-conditioned soils under the two inoculum addition treatments were compared to those measurements of seedlings grown in mixture under the same treatment conditions (i.e. the two inoculum addition treatments that did not receive acacia-topsoil). This was done in order to determine whether competition was occurring between the *A. saligna* and *P. pinnata* seedlings and whether the addition of exotic *Bradyrhizobium* strains (i.e. familiar association) favored the competitiveness of *A. saligna*. These comparisons were done using two approaches. First, in order to determine performance of each species under the different competition treatments (i.e. grown alone vs in mixture), the raw growth performance and BNF data were compared using a generalized linear mixed model (Gaussian distribution, link = "identity") with the *lme* function in the *nlme* R package with 'Australian inoculum addition' and 'competiton treatment', and their interaction, as fixed effects (Pinheiro *et al.*, 2013). A random factor ('transplanted') was included to account for potential differences due to transplanting of seedlings in pots where initial germination failed. Overall effect sizes and their significance were determined using the *anova* function in the R base package. Pairwise contrasts between levels of the fixed effects were determined using the *emmeans* function in the *emmeans* R package (Lenth *et al.*, 2018). Additionally, in order to determine whether the δ^{15}N values for each growth setup by inoculum addition treatment combination was significantly different from zero, I used a one-sample t-test (mu=0) or a one-sample Wilcoxin test (mu=0) for parametric and non-parametric groups, respectively. This was repeated for both species.

Second, in order to determine the level of competition between the two species, the Relative Competition Index (RCI; (Weigelt & Jolliffe, 2003) was calculated for each species using the following equation:

$$RCI = \frac{P_{alone} - P_{mix}}{P_{alone}}$$

where P_{alone} is the average performance of the seedlings grown alone in a pot and P_{mix} is the performance of the same species grown with the other species in a single pot, both of which were subjected to the same inoculum addition treatment. Because pots containing seedlings

grown alone and those grown in mixture were not paired, the average performances for seedlings grown alone was calculated for each species by inoculum addition treatment combinations to calculate RCI values. This average value for P_{alone} remained fixed in the equation while the different values for the seedlings grown in mixture were used as P_{mix}. This was done for each species by inoculum addition treatment combination. Additionally, in order to calculate the RCI value δ^{15}N, values were rescaled to be positive by adding the absolute value of the lowest recorded δ^{15}N measurement to all other δ^{15}N measurements. The resultant RCI values were used as the response variable in a generalized linear mixed model (Gaussian distribution, link = "identity") using the *lme* function in the *nlme* package in order to determine the effect of Australian inoculum addition on competition between the two species. 'Australian inoculum addition' and 'host species', as well as their interaction, were included as fixed effects, and 'pot' was included as a random effect. Overall effect sizes and their significance were determined by type I sum of squares using the *anova* function in the R base package for measurements of seedling height, seedling shoot dry biomass and δ^{15}N as the order of the fixed effects of the model did not alter the outcome. Effect sizes for measurements of seedling root dry biomass and nodule numbers were determined by type III sum of squares using the *Anova* function in the *car* R package due to the significant interaction between the two main effects (host species and inoculum addition) (Langsrud, 2003; Macnaughton, 1998). Pairwise contrasts between levels of the fixed effects were determined using the *emmeans* function in the *emmeans* R package.

RCI values give an indication of how plants grown alone perform relative to when grown under competition. More specifically, this metric determines performance in the absence of competition relative to the proportional reduction (or increase) in performance when grown under competition. A positive RCI value (RCI>0) indicates that plants grown in mixture perform worse than those grown alone, thus competition is occurring. A negative RCI value (RCI<0) indicates that plants grown in mixture are performing better than those grown alone, thus facilitation is occurring between plants grown in mixture. RCI values for δ^{15}N are interpreted in the opposite manner to the other growth performances as, in this case, lower values are indicative of higher BNF. Therefore, a positive RCI for δ^{15}N would indicate that δ^{15}N values were lower, and thus BNF was higher, for those seedlings grown in mixture compared to when grown alone, and *vice versa*. For all performance and BNF measures, a RCI value of zero (RCI=0) indicates that plants grown in mixture are performing identically to those

grown alone and, thus, no interaction is occurring between plants grown in mixture. Therefore, in order to determine whether the RCI values for each species by inoculum addition treatment combination was significantly different from zero, I used a one-sample t-test (mu=0) or a one-sample Wilcoxin test (mu=0) for parametric and non-parametric data distributions, respectively.

In order to determine the relative contribution of Australian inoculum and acacia topsoil addition on the competitive ability of *A. saligna*, I compared growth performances and BNF measures for seedlings of both legumes grown in mixture under the four inoculum by topsoil addition treatment combinations. This entailed initial analyses to assess how each treatment combination effected the performances of each species separately. These were done using generalized linear mixed models with a Gaussian distribution (link = "identity") (*lme* function in *nlme* package) with 'Australian inoculum addition' and 'topsoil addition', as well as their interaction, as main effects, and with 'transplanted' included as a random effect, for each legume separately. Significance of each main effect was determined using the *anova* function (type I sum of squares) of the R base package as the variable order within the model did not alter the outcome. Additionally, in order to determine whether the δ^{15}N values for each inoculum addition by topsoil addition treatment combination was significantly different from zero, I used a one-sample t-test (mu=0) or a one-sample Wilcoxin test (mu=0) for parametric and non-parametric groups, respectively. This was repeated for both species. Furthermore, in order to determine the impact of each treatment combination on the performance of *A. saligna* relative to *P. pinnata*, the relative performance of *A. saligna* was calculated per pot under the competition treatment (i.e. grown in mixture only) as:

$$Relative\ P_{acacia} = \frac{P_{acacia}}{P_{acacia} + P_{psoralea}}$$

where P_{acacia} represents the performance measurement for *A. saligna* and $P_{psoralea}$ represents the performance measurement for *P. pinnata*. δ^{15}N values were made positive for these four treatment combinations following the same protocol as for calculations of RCI values. The relative performance values were used as the response variable in a factorial analysis of variance (ANOVA), with 'Australian inoculum addition' and 'topsoil addition', as well as their interaction, as main effects, followed by a Tukey HSD post-hoc test.

DNA extraction, next-generation sequencing of root nodule bacteria and bioinformatics

In order to determine whether the seedlings of both legumes were forming familiar or novel rhizobial associations, as well as whether they were competing for rhizobial associations, next generation sequencing (NGS) methods were used to identify the rhizobia within their root nodules. The same DNA extraction, NGS and bioinformatic protocols were followed as specified in Chapter 2. In summary, nodules from seedlings grown in mixture were pooled for each treatment combination separately for each species (4 treatment combinations x 2 host species = 8 samples). DNA was extracted from these nodules using the DNeasy® Plant Mini Extraction Kit (Qiagen, supplied by White Head Scientific, Cape Town, South Africa) according to the manufacturer specifications. The nodulation C (*nodC*) gene was amplified for NGS, using the primers nodCF12F (5'-CCG GAT AGG MTG GKB CCR TA-3') and nodCRI2R (5'-GTG CAC AAS GCR TAD RCC TTC AH-3'), with sample-specific barcodes in the forward primer. Sequencing was performed by the Molecular Research LP next-generation sequencing service (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument following manufacturer protocols. The resultant sequences were quality filtered, cleaned and trimmed and clustered to a 97% similarity level. Sequences were blasted against the NCBI's GenBank database (http://blast.ncbi.nlm.nih.gov/Blast) to determine taxonomic identity. All non-nitrogen-fixing bacteria were removed from the dataset so that only rhizobial strains were considered in subsequent analyses. Lastly, all rare OTUs with a relative abundance <5% per sample for all samples were removed.

Results

Contribution of exotic Bradyrhizobium to competition dynamics of A. saligna *and* P. pinnata

For both legumes, comparisons between competition and inoculum addition treatment combinations indicated that competition treatment was a significant predictor for the majority of growth performance and BNF measures. Seedlings often displayed increased performance when grown alone rather than in mixture. *Psoralea pinnata* seedling height is the only exception as, in this case, seedlings grew to similar heights regardless of competition or inoculum treatments (Table 3.1 and Fig. 3.1), although there was a non-significant trend of reduced height under inoculum addition when grown alone. Overall, there were no significant inoculum effects for either species (Table 3.1), however, there was a significant competition by inoculum addition interaction effect for *A. saligna* root biomass ($F_{(1, 27)}$ = 11.7383; p = 0.0002). Specifically, when grown alone, *A. saligna* root biomass was significantly lower under

inoculum addition treatment compared to plants grown with no inoculum addition (Fig. 3.1). *Acacia saligna* nodule numbers also differed between plants grown alone and in mixture, but only for those seedlings that received no inoculum. Specifically, nodule numbers were lower for seedlings grown in mixture than those grown alone. δ^{15}N values did not differ between the four treatment combinations for either legume species. However, δ^{15}N values was equal to, or significantly lower than, zero only for *P. pinnata* (i.e. BNF was taking place), while *A. saligna* δ^{15}N values were all significantly greater than zero (Fig. 3.1).

Comparisons of RCI values for different growth performance and BNF measures showed varying results (Fig. 3.2; Table 3.2). No competitive effect was detected for seedling height of *P. pinnata*, while the height of *A. saligna* seedlings was significantly reduced by competition with *P. pinnata*, indicated by RCI values being zero and significantly positive, respectively. However, this maintenance of *P.pinnata* seedling height under both competition treatments (i.e. when grown alone and in mixture) did not translate into shoot biomass. For the latter, both species had significantly positive RCI values, i.e. both experienced a reduction in shoot biomass when grown in mixture, and these did not significantly differ between the inoculum addition treatments. There was a significant interaction between species and inoculum addition for seedling root biomass ($\chi^2_{(1)}$ = 13.506; p = 0.0002) and nodule numbers ($\chi^2_{(1)}$ = 4.5046; p = 0.0338) RCI values (Table 3.2). These values did not differ between inoculum addition treatments for *P. pinnata* and were significantly positive. Contrary, there was a significant difference between the two inoculum treatments for *A. saligna* seedlings with RCI values being significantly positive only for those seedlings that received no inoculum while RCI values equalled zero for *A. saligna* seedlings that received inoculum (Fig. 3.2). However, for *A. saligna* root biomass, this apparent beneficial inoculum effect should be viewed with caution due to low root biomass accumulation of seedlings grown alone under inoculum addition compared to when no inoculum was added (Fig. 3.1), i.e. *A. saligna* seedlings were not out-competing *P. pinnata* when innoculated, but simply accumulating root biomass as poorly when grown alone as when grown in mixture. *Acacia saligna* RCI values for nodule number show that in the presence of Australian inoculum it experienced no interaction with *P. pinnata* when grown in mixture, while seedlings experienced competition in terms of nodule number when inoculum was not added. These results are matched by those seen for the raw nodule number comparisons in Figure 3.1. On the other hand, *P pinnata* was

experiencing competition with *A. saligna* in terms of nodule number, regardless of the addition of inoculum or not.

There was a significant effect of inoculum addition on the RCI values of δ^{15}N ($F_{(1, 18)}$ = 4.6785; p = 0.0442) (Table 3.2), indicated by significantly positive RCI values for both legume species when inoculum was added compared with when no inoculum was added (RCI values equal zero). In order to calculate RCI values for BNF, δ^{15}N values were rescaled by adding the absolute value of the lowest δ^{15}N value to all datapoints. As such, positive RCI values indicate that plants grown in mixture are more reliant on BNF, than those grown alone (which could indicate competition for soil available nitrogen). For both species, there was a non-significant but decreasing trend in raw δ^{15}N values between the competition treatments with those seedling grown in mixture having slightly lower δ^{15}N values than those grown alone (Fig. 3.1). This trend is more prominent for those seedlings that had received Australian inoculum as shown by the significantly positive RCI values for this treatment (Fig. 3.2). However, differences between the inoculum treatments for both species were non-significant (Fig. 3.2) as well as between the four competition and inoculum addition treatment combinations (Fig. 3.1).

Relative contribution of acacia topsoil addition and Australian inoculum addition to competition dynamic

Comparisons between topsoil and Australian inoculum addition treatment combinations for each species were non-significant for most of the different growth performance and BNF measures (Fig. 3.3). However, topsoil was a significant fixed effect for *A. saligna* seedling height ($F_{(1, 28)}$ = 4.2912; p = 0.0476) and *P. pinnata* shoot biomass ($F_{(1, 28)}$ = 6.2117; p = 0.0189) (Table 3.3). In both cases, the addition of topsoil tended to increase the growth performances, though these were non-significant between the four treatment combinations. δ^{15}N values do not differ between the four treatment combinations, however, values were significantly positive for *A. saligna* (indicating low or no BNF) and values are not significantly different to zero for *P. pinnata* (indicating BNF is likely occurring). There was a tendency for *A. saligna* seedlings to have higher relative performance when inoculum was added without topsoil. However, these were non-significant. In fact, results of the comparisons of the relative growth performances and BNF measurements of *A. saligna* revealed no significant differences between the four topsoil addition and inoculum addition treatment combinations (Table S3.2; Fig. S3.1).

NGS bioinformatics and OTU abundances

After data quality-checking my *nodC* dataset generated 280 zOTUs. Clustering of these at 97% DNA similarity level, followed by the removal of singleton/doubleton OTUs, OTUs representing non-fixing bacteria, and OTUs with <5% relative abundance across all samples, resulted 890,680 sequences representing 10 OTUs for all rhizobia.

Blast results for the 10 OTUs indicated that the majority of them belonged to the genus *Bradyrhizobium* (6 OTUs) followed by *Mesorhizobium* (4 OTUs) (also see Chapter 2 and Table S2.5). Reference samples used as the Australian *Bradyrhizobium* inoculum cocktail were dominated by two OTUs (i.e. with relative abundances >5%), SW OTU1 and SW OTU6. These blasted to *Bradyrhizobium* sp. CPI 240 and *Bradyrhizobium* sp. CPI241, respectively, which were previously isolated from *Acacia* spp. in Australia (Barrett *et al.*, 2016). While there was some overlap in associations with rarer OTUs between both legumes, SW OTU1 and SW OTU2 were by far the dominant strains isolated from nodules of *A. saligna* and *P. pinnata*, respectively, with blast results identifying the latter as *Mesorhizobium* sp. 969n9 previously isolated from South African legumes (Lemaire & Muasya, unpublished; Fig. 3.4). *Acacia saligna* also appeared to associate with a higher diversity of *Bradyrhizobium* strains while *P. pinnata* largely associated with one strain (SW OTU2). *Psoralea pinnata* also associated with several *Bradyrhizobium* OTUs, particularly SW OTU1, when grown in mixture under the no topsoil added x Australian inoculum added treatment combination (Fig. 3.4). Associations with SW OTU2 were, nonetheless, still dominant for these seedlings and no negative effects of the association with *Bradyrhizobium* on growth were detected from the growth performance and BNF comparisons (Fig. 3.3).

Discussion

The high invasiveness of Australian acacias in the CCR has been attributed to their association with bradyrhizobia, as well as their ability to quickly generate positive-feedbacks through increased leaf litter inputs (Gaertner *et al.*, 2014; Le Maitre *et al.*, 2011; Morris *et al.*, 2011). In the CCR, these acacias frequently associate with bradyrhizobia of Australian origin (Ndlovu *et al.*, 2013; Warrington *et al.*, 2019) which may further enhance these feedbacks (Le Roux et al., 2017). While I found evidence suggesting that invasive *A. saligna* and native *P.*

pinnata are competing, there was little indication that *A. saligna* is the stronger competitor, nor that the competitive performance of this species is enhanced by either Australian bacteria or the onset of *Acacia*-induced abiotic soil conditions. Specifically, both *A. saligna* and *P. pinnata* experienced similar reductions in performance when grown in mixture compared to when grown alone (Figs. 3.1 and 3.2). The relative performances of *A. saligna* were also similar across all *Acacia*-topsoil and Australian inoculum addition treatment combinations with only a non-significant trend of Australian inoculum addition having a facilitatory effect. Therefore, I partially reject my hypothesis that the presence of familiar associations would facilitate the performance of *A. saligna* relative to *P. pinnata* and that the presence of both Australian inoculum and *Acacia*-topsoil would act synergistically to facilitate *A. saligna* with concomitant negative impacts on the performance of *P. pinnata*.

NGS barcoding showed that each species associated with a distinct subset of the soil rhizobial community, with *A. saligna* forming associations predominantly with *Bradyrhizobium* strains of Australian origin (i.e. SW OTU1; also see Fig. 2.5 in Chapter 2), while *P. pinnata* associated predominantly with *Mesorhizobium* strains (i.e. SW OTU2; Fig. 3.4). The two rhizobium genera represent the known and preferred rhizobial partners of each legume (*Acacia* – Keet *et al.*, 2017; Lafay & Burdon, 2001; Marsudi *et al.*, 1999; Rodríguez-Echeverría, 2010; Le Roux *et al.*, 2018; Warrington *et al.*, 2019; *Psoralea* – Kanu & Dakora, 2012; Lemaire *et al.*, 2015; Stirton *et al.*, 2015). Additionally, *A. saligna* associated with a number of *Bradyrhizobium* strains of non-Australian origin (i.e. 'Other' in Fig. 3.4) in these *Psoralea*-conditioned soils. Blast results showed that the majority of these strains had been isolated from invasive *Acacia dealbata* in the United States of America (Table S2.5 in Chapter 2) (Urbina & Klock, unpublished), therefore it is plausible that these strains had been co-introduced from Australia. Therefore, while the addition of Australian *Bradyrhizobium* inoculum did not improve *A. saligna*'s competitive ability relative to *P. pinnata*, the formation of distinct but familiar associations by both species undoubtedly contributed to their similar performances when grown in mixture.

In previous studies, the presence and enrichment of acacia-associated *Bradyrhizobium* strains has been shown to have a direct competitive effect on native rhizobia, inhibiting their ability to nodulate their native legume hosts (e.g. Rodríguez-Echeverría *et al.*, 2012). Therefore, it is striking that the distinct rhizobial associations I observed for *A. saligna* and *P.*

pinnata remained regardless of whether the competition treatment as well as the addition of Australian *Bradyrhizobium* strains or acacia topsoil. This suggests that both species have high rhizobium specificity and selectivity, and that native and exotic bacteria potentially co-exist in CCR soils. CCR legumes and the composition of the belowground biotic communities are tightly linked (Slabbert, Kongor, Esler, *et al.*, 2010), and previous studies have found high compositional turnover between rhizobia associating with native CCR legumes and co-ccurring invasive Australian acacias (Le Roux *et al.*, 2016). However, these authors also found that the rhizobia associating with CCR legumes were compositionally different between uninvaded and invaded sites, suggesting that acacias do impact associations for some CCR legumes (Le Roux *et al.*, 2016). Therefore, *P. pinnata*'s association with its preferred *Mesorhizobium* strains despite the presence of *A. saligna* and exotic bradyrhizobia may be an exception rather than the rule. The formation of familiar associations for *P. pinnata*, as well as *A. saligna*, in all cases would explain the practically neglible Australian inoculum addition effect on both inhibiting *P. pinnata* performances and facilitating *A. saligna* growth. One has to consider the possibility that the lack of an inoculum effect is due to the cross-contamination of pots during the glasshouse experiment. Several considerations suggest that both these scenarios are unlikely. These are laid out in detail in Chapter 2 (see Discussion on page 35) and include, briefly, the stringent protocols put in place during soil collections and glasshouse experiments, the previously documented presence of Australian *Bradyrhizobium* within the CCR (Ndlovu *et al.*, 2013; Warrington *et al.*, 2019) as well as similarities in SW OTU *nodC* sequences with those isolated from previous studies (Fig. 2.5) (Keet *et al.*, 2017; Le Roux *et al.*, 2018). Furthermore, *Psoralea*-conditioned soils used in this chapter were the same as those used in Chapter 2 (Table S2.1 and Table S3.1). Therefore, the most parsimonious explanation is that Australian bradyrhizobia were already present within these soils.

I found a significant inoculum effect for seedling root biomass RCI values (Fig. 3.2), suggesting that the presence of Australian inoculum facilitated the competitive ability of *A. saligna*. However, closer inspection of the raw data revealed that this positive effect was only due to seedlings having exceptionally low root biomass when grown alone when inoculum was added (Fig. 3.1). The lower investment in root biomass when grown alone may be driven by the association with effective *Bradyrhizobium* strains, as this often leads to less root development and increased shoot biomass i.e. less root biomass is required to forage for nitrogen from the soil (Poorter & Nagel, 2000; Rodríguez-Echeverría *et al.*, 2009). However,

this is unlikely to be the case since a non-significant trend was also present for *A. saligna* shoot biomass under the same treatment combination, and δ^{15}N values of *A. saligna* suggest that it was not wholly utilizing BNF for nutrient assimilation. Therefore, these observations do not support the addition of inoculum as being facilitative to acacia performance, but rather that some other factor was at play to decrease root biomass in the presence of inoculum. It could be that some component of the inoculum itself was responsible for the reduction in root biomass accumulation, however, this is challenging to unravel due to the inconsistency of the negative effect across growth performance measures and across species. There was also a significant inoculum effect for *A. saligna* nodule numbers which tended to increase under inoculation (Fig. 3.1; Table 3.1). In the absence of Australian inoculum, nodules numbers of seedlings grown in mixture were significantly lower than those of seedlings grown alone, while those seedlings that received Australian inoculum maintained similar nodule numbers. However, considering that the rhizobia associating with *A. saligna* under all four treatment combinations are the same *Bradyrhizobium* strains, which were also not utilized by *P. pinnata*, it is likely that this reduction in nodule number is simply due to a lower root biomass, and therefore lower rhizobial encounter rates (Ramoneda *et al.*, 2020), for *A. saligna* seedlings grown in mixture rather than competition for effective rhizobial associations. The facilitation of inoculum addition for increased nodule number under mixture is likely due to those seedlings that received inoculum having increased *Bradyrhizobium* availability.

Plant-plant competition is often most intense at the seedling stage, the outcome of which is largely dependent upon the nutrient acquisition strategies of species (Witkowski, 1991). Nutrient acquisition is influenced by three major factors: root structure, ability to form nutrient-acquiring mutualistic associations, and soil nutrient availability (Lambers, Mougel, Jaillard, *et al.*, 2009). In terms of the competitive dynamics between *A. saligna* and *P. pinnata*, similar growth performances across all treatment combinations when grown under competition would suggest that they possess equally effective nutrient acquisition capabilities, at least during the early stages of seedling development investigated here (Fig. 3.1). This similarity in competitive ability for nutrient acquisition may be due to several factors. Firstly, there was no competition for rhizobial associations between the two host species as they associated with distinct subsets of available rhizobial mutualists (Fig. 3.4). Furthermore, δ^{15}N values suggest that *A. saligna* was less reliant on BNF (and primarily utilizing soil nitrogen; $\delta^{15}N>0$), while *P. pinnata* appeared to rely mostly on BNF for its nitrogen requirements ($\delta^{15}N\leq0$; Unkovich,

2013). This was the case regardless of competition (alone vs in mixture), inoculum or topsoil addition (Fig. 3.1 and Fig. 3.3). *Psoralea pinnata*, like most CCR legumes, is capable of increasing soil nitrogen availability (Chimphango *et al.*, 2015; Stirton *et al.*, 2015). While the association with rhizobia allows *A. saligna* to thrive in low nutrient environments, comparisons with other native CCR species, such as *Protea repens*, have shown that it is more efficient at acquiring nutrients when soil nutrient levels are high (Witkowski, 1991). This superior competitive ability of *A. saligna* in soil nutrient acquisition is unsurprising considering that the species originates from some of the most nutrient poor environments in the world (Young & Young, 2001). However, there was a non-significant decrease in δ^{15}N values for both species when grown in mixture and, for *A. saligna*, this may suggest that as soil nutrients become increasing depleted it would become more reliant on BNF to meet its nutrient requirements. While this resource partitioning is the most likely explanation for the apparent co-existence of *A. saligna* and *P. pinnata*, it is plausible that the energetic expenses of BNF, which is more carbon-costly than soil nitrogen assimilation (Graham, 1992), may result in long-term negative impacts on *P. pinnata* growth performances should the soil nitrogen pool remain undepleted (e.g. through leaf litter addition or through nodule nitrogen deposition; Yelenik *et al.*, 2004).

Aside from rhizobium symbiosis, acacias can also modify soil abiotic conditions to create positive feedbacks (Le Maitre *et al.*, 2011; Yelenik *et al.*, 2004), such as the release of allelopathic chemicals that inhibit native species (Abd El-Gawad & El-Amier, 2015). I found no evidence to suggest a negative allelopathic effect, via *Acacia*-topsoil, on both legumes included in this study. Rather, I found a marginal increasing trend in growth performances for both species for measures such as seedling height and shoot biomass under topsoil addition (Fig. 3.3). Similarly, Yannelli, Novoa, Lorenzo, *et al.* (2020) recently found allelopathic chemicals of some invasive acacias to increase germination and seedling growth of other acacias and to not negatively effect native CCR species. Evidence in another Mediterranean region, the North-west Iberian Peninsula, demonstrated that altered microhabitats i.e. local soil conditions, by well-established acacia populations, rather than their allelopathic chemicals, were responsible for facilitating seed germination and seedling growth of non-native acacias as well as native species (Lorenzo, Rodríguez, González, *et al.*, 2017), further suggesting that soil conditioning by acacias are significant for their establishment success. However, it is important to keep in mind that invader-induced abiotic changes, and the biotic responses to them, act simultaneously rather than sequentially (Hobbs, Higgs & Harris, 2009) and that these

changes predominantly manifest over long time periods (Yelenik *et al.*, 2004). The negative effects of *A. saligna* allelopathic chemicals have been previously documented (Abd El-Gawad & El-Amier, 2015). Therefore, it is plausible that the duration of my experiments were too short to represent a truly accurate account of *Acacia*-topsoil effect on plant competition under field conditions i.e. when acacia stands are dense and dominating an area. Another possibility is that the acacia topsoil used in my experiments was not sufficient to capture the effects of allelopathy as these chemicals may not have persisted in these soils or may have degraded after the sterilization step (see Methods page 57).

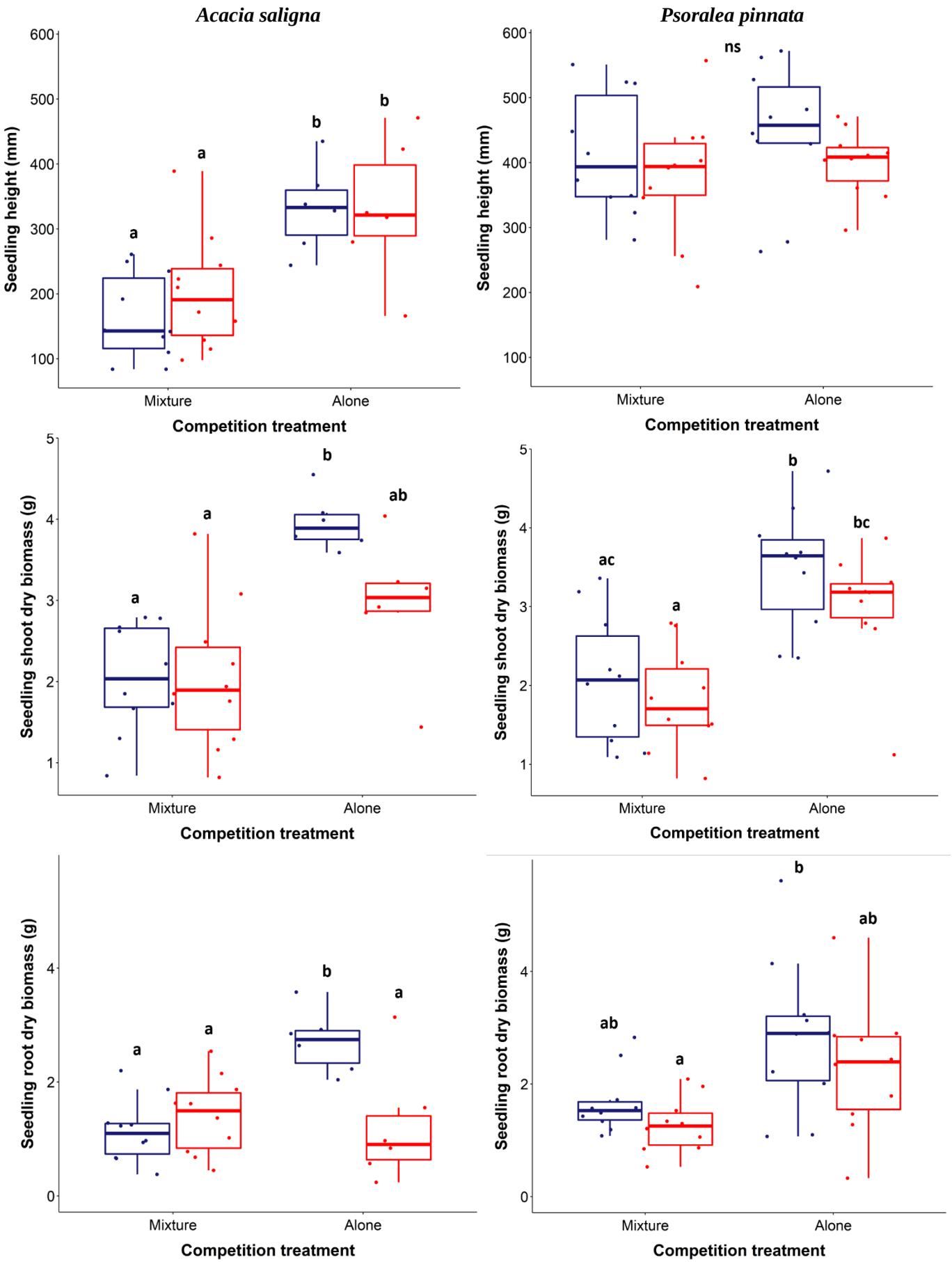
The similar competitive abilities of *A. saligna* and *P. pinnata* may also be due to them sharing several similar functional traits. *Psoralea* species are able to nodulate efficiently with cosmopolitan *Mesorhizobium* strains and thus are able to efficiently acquire nitrogen in low-nutrient environments (Kanu & Dakora, 2012). *Psoralea pinnata* can withstand soil temperatures similar in range to *A. longifolia* (Behenna, Vetter & Fourie, 2008) and exhibits a similar post-fire germination strategy to *A. saligna*. That is, large numbers of seeds are deposited that germinate post-fire in dense, monotypic groups of fast-growing seedlings where competition for resources, such as light, is intense (Stirton *et al.*, 2015). These increases in shoot length under limiting conditions were also evident in this experiment where seedlings often grew taller than those of *A. saligna* under competition (Fig. 3.2). In the field, adult shrubs/trees can also vary in height from 2m to 7m tall, thus successfully shading co-occurring native shrubs (similarly to *A. saligna*; Witkowski, 1991). Additionally, their association with nitrogen-fixing rhizobia results in *Mesorhizobium*-enrichment and subsequent increases in soil nitrogen availability (Chimphango *et al.*, 2015; Stirton *et al.*, 2015). In fact, these functional traits have probably contributed to the successful naturalization of the species in Australia in areas where *A. saligna* naturally occurs (Stirton *et al.*, 2015). *Psoralea pinnata*'s resilience in the face of *Acacia* invasion is also evident in restoration projects of riparian zones within the CCR, as it is one of the few native species to regenerate unassisted following the clearing of acacia thickets (Reinecke *et al.*, 2008). Together with these observations, the ability of *P. pinnata* to successfully sanction exotic *Bradyrhizobium* associations in favor of its preferred *Mesorhizobium* strains, as well as the co-existence evident between *Mesorhizobium* and *Bradyrhizobium* strains in CCR soils, suggests that *P. pinnata* may be a good candidate for active restoration following acacia clearing (Funk, Cleland, Suding, *et al.*, 2008).

While this study found no major facilitatory effects of *Bradyrhizobium* inoculum or topsoil addition for the competitive ability *of A. saligna*, seedlings were nonetheless able to perform similarly to *P. pinnata* seedlings, regardless of the fact that all soil conditions were skewed in favor of the latter (i.e. soils were pre-conditioned by well-established *P. pinnata* populations). This is a testament to the ecological flexibility of *A. saligna* and highlights the species' resource-acquisition trait flexibility to exploit the most energy-efficient nutrient pools available to it (Morris *et al.*, 2011; Witkowski, 1991; Yelenik *et al.*, 2004). While recent studies have shown that changes to soil abiotic conditions by well-established acacia populations facilitate seedling establishment and are conducive to acacia nodulation by *Bradyrhizobium*, the presence of familiar Australian *Bradyrhizobium* are nonetheless more important at the early-establishment/seedling stage prior to these soil changes (Le Roux *et al.*, 2018; Wandrag *et al.*, 2013). The presence of these exotic *Bradyrhizobium* in *Psoralea*-conditioned soils is cause for concern as it suggests that the limitations to establishment imposed by symbiont availability may no longer apply to *A. saligna*, or acacias in general (Keet *et al.*, 2017), in CCR soils (Chapter 2; Wandrag *et al.*, 2020). In this study, *A. saligna* was associating with *Bradyrhizobium* strains even though it was not making use of BNF for its nitrogen requirements, yet its growth performances in a competition scenario remained on par with *P. pinnata*. This may suggest that this association is highly efficient in terms of carbon-costs to *A. saligna* hosts, thus potentially allowing this species to hedge its bets (e.g. Sadeh *et al.*, 2009) in the event that soil nutrients are depleted and it would once again rely on rhizobia.

Generally, competition for resources is dependent upon the characteristics of both the invaded region and the invader's biological traits (Thuiller, Richardson, Rouget, *et al.*, 2006). The findings of this study suggest that there is not a strong competitive dominance of *A. saligna* over *P. pinnata*, which aligns well with the observations of *P. pinnata* persistence in *Acacia*-invaded areas. Additionally, there is limited evidence of a facilitatory effect of positive feedbacks induced by *Acacia*-topsoil on acacia competitive ability over *P. pinnata*. The distinct rhizobial associations as well as similarities in functional traits, such as increased growth rates, of these two legumes are likely responsible for the similarities in their competitive abilities. Moreover, it appears that the most important factor facilitating the co-existence between these two species is nitrogen niche divergence, whereby *A. saligna* is assimilating soil nitrogen while *P. pinnata* is utilizing atmospheric nitrogen through BNF. While these species may be competing for other resources, such as light, they are not competing for this vital and plant

growth-limiting nutrient. However, this is often not the case with other native CCR legumes which have experienced altered rhizobial associations in acacia-invaded sites (Le Roux *et al.*, 2016). Non-leguminous plants are also outcompeted by acacias due to their slower growth rates and low-nutrient adaptations (Witkowski, 1991). Additionally, my results suggest that Australian *Bradyrhizobium* strains appear to be naturally widespread in CCR soils (also see Keet *et al.* (2017) and Le Roux *et al.* (2018)), thereby potentially enhancing the competitive ability and establishment success of introduced acacias. This highlights the high context-dependency surrounding non-native plant invasions (Novoa, Richardson, Pyšek, *et al.*, 2020). Future studies should consider investigating the competitive dynamics between acacias and CCR legumes nodulated by native strains that are less cosmopolitan than *Mesorhizobium,* as this may alter the dynamics of competition between native rhizobia and Australian bradyrhizobia for associations with native legumes and acacias. Furthermore, this should be done within a variety of soil types/nutrient conditions (e.g. invaded vs uninvaded) in order to determine the role of each nutrient acquisition pool (atmospheric vs soil nitrogen) in conferring competitive superiority to acacias or competing native species.

Tables and Figures



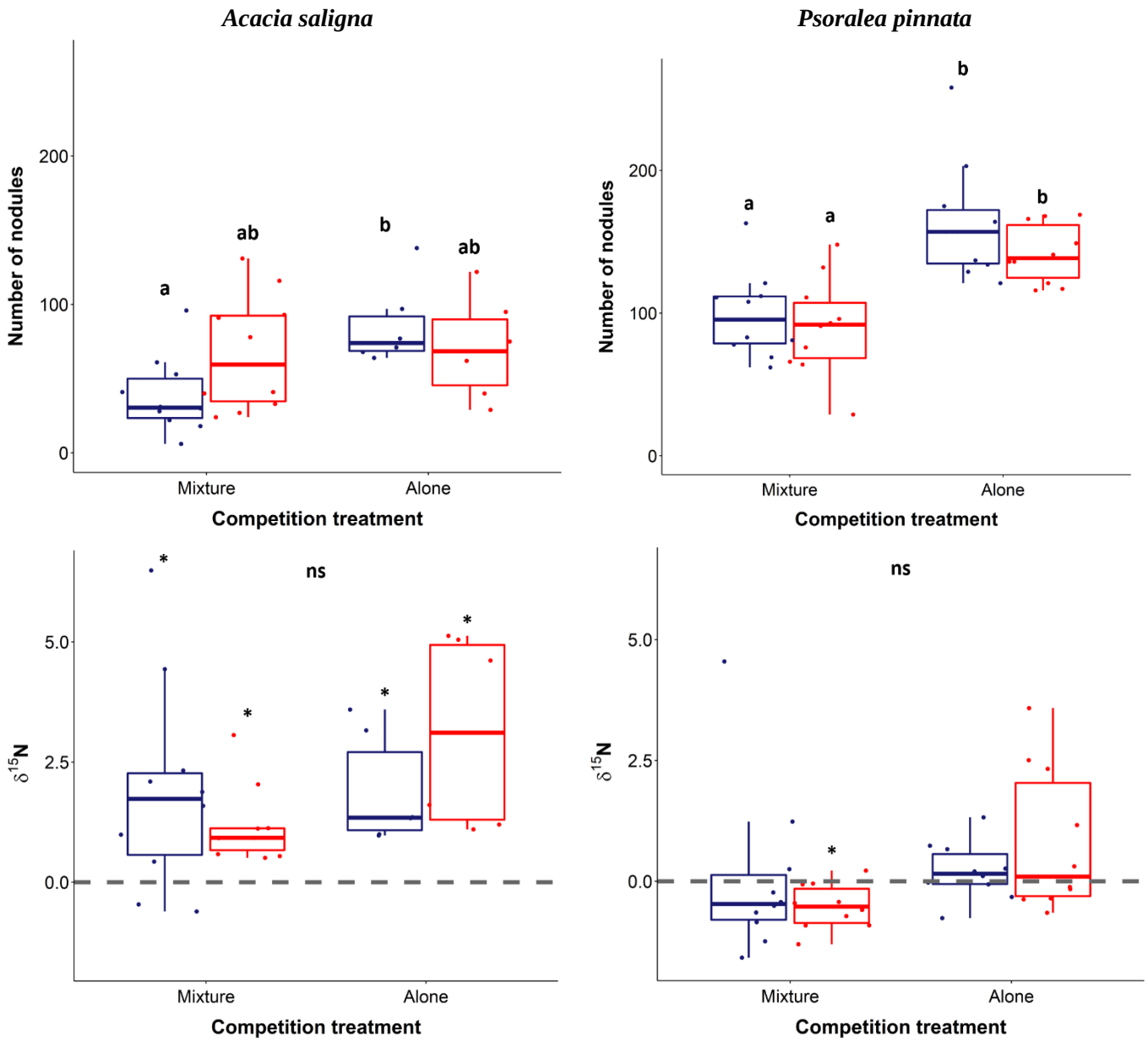


***Figure 3.1**: Growth performance (seedling height, seedling shoot dry biomass and seedling root dry biomass) and BNF (number of nodules and $\delta^{15}N$) measurements for* Acacia saligna *(left) and* Psoralea pinnata *(right) for each competition treatment (grown alone; grown in mixture) by inoculum treatment (red – Australian inoculum added; blue – no inoculum added) combination. The broken horizontal line in the $\delta^{15}N$ graphs indicates where $\delta^{15}N = 0$. The * indicates which $\delta^{15}N$ values for each growth setup by inoculum treatment combination is significantly different to zero.*

***Table 3.1**: Results of ANOVAs of fixed effects for generalized linear mixed models comparing the different growth performance and BNF measurements between different competition treatment (grown alone/grown in mixture) and inoculum addition treatment (inoculum added/no inoculum) combinations for* Acacia saligna *and* Psoralea pinnata.

		Acacia saligna				***Psoralea pinnata***			
		numDf	denDf	F-value	p-value	numDf	denDf	F-value	p-value
Seedling shoot length	Intercept	1	27	266.0608	<0.0001	1	35	152.0537	<0.0001
	Inoculum addition	1	27	0.6629	ns	1	35	2.0432	ns
	Competition treatment	1	27	24.0333	<0.0001	1	35	0.4483	ns
	Inoculum addition: Competition treatment	1	27	0.4377	ns	1	35	0.054	ns
Seedling shoot biomass	Intercept	1	27	132.939	<0.0001	1	35	26.6852	<0.0001
	Inoculum addition	1	27	2.212	ns	1	35	2.5925	ns
	Competition treatment	1	27	26.1014	<0.0001	1	35	24.1688	<0.0001
	Inoculum addition: Competition treatment	1	27	3.3836	ns	1	35	0.2574	ns
Seedling root biomass	Intercept	1	27	152.8532	<0.0001	1	35	172.2946	<0.0001
	Inoculum addition	1	27	2.5054	ns	1	35	2.3936	ns
	Competition treatment	1	27	7.1557	<0.0001	1	35	12.4198	0.0012
	Inoculum addition: Competition treatment	1	27	11.7383	0.002	1	35	0.0597	ns
Number of nodules	Intercept	1	27	117.0925	<0.0001	1	35	117.1628	<0.0001
	Inoculum addition	1	27	1.1268	ns	1	35	2.1393	ns
	Competition treatment	1	27	4.4586	0.0441	1	35	28.5537	<0.0001
	Inoculum addition: Competition treatment	1	27	3.4279	ns	1	35	0.4326	ns
$\delta^{15}N$	Intercept	1	27	2.4477	ns	1	35	0.3014	ns
	Inoculum addition	1	27	0.3001	ns	1	35	0.00021	ns
	Competition treatment	1	27	5.6166	0.0252	1	35	4.7817	0.0355
	Inoculum addition: Competition treatment	1	27	2.125	ns	1	35	2.4709	ns

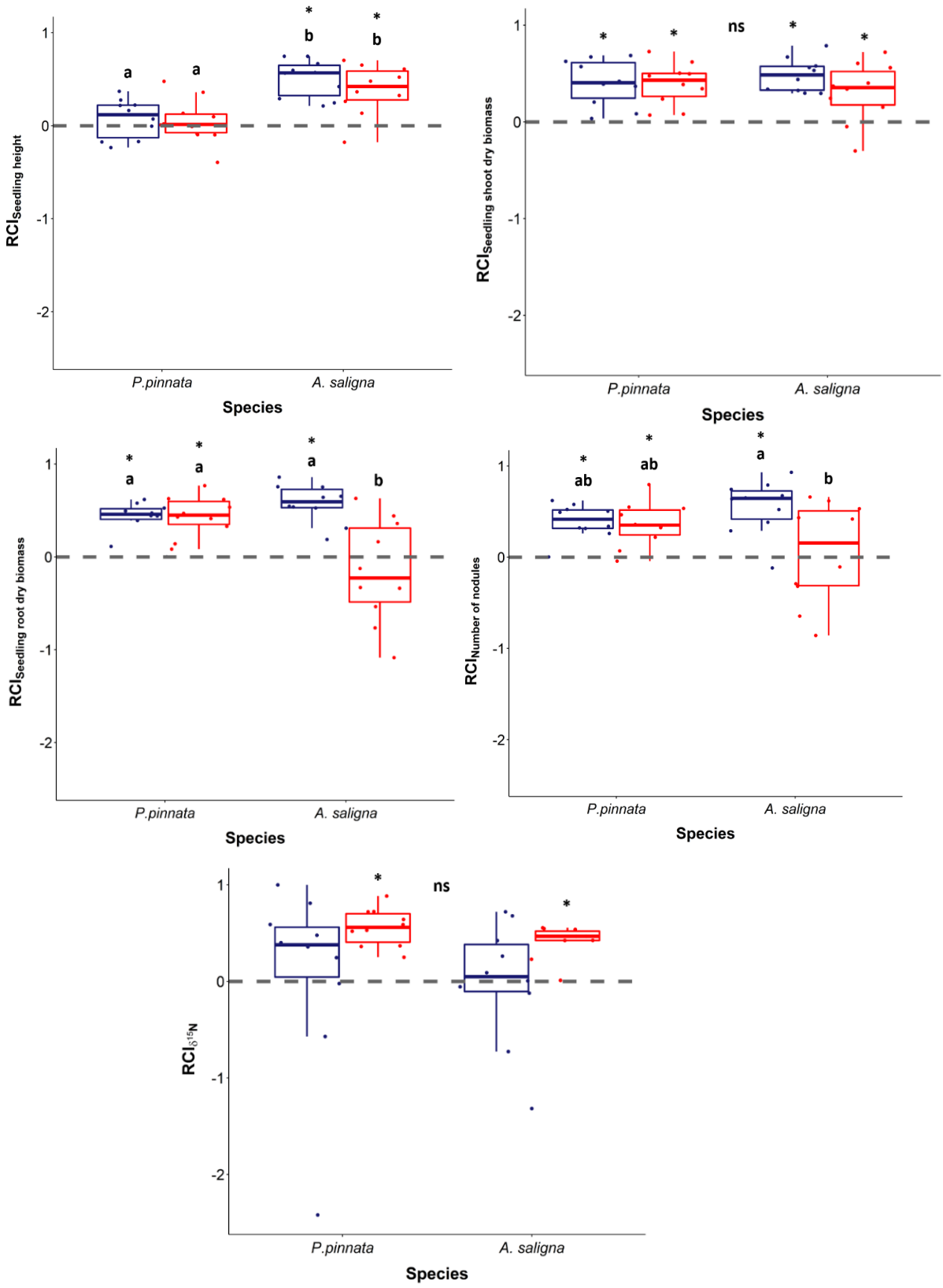
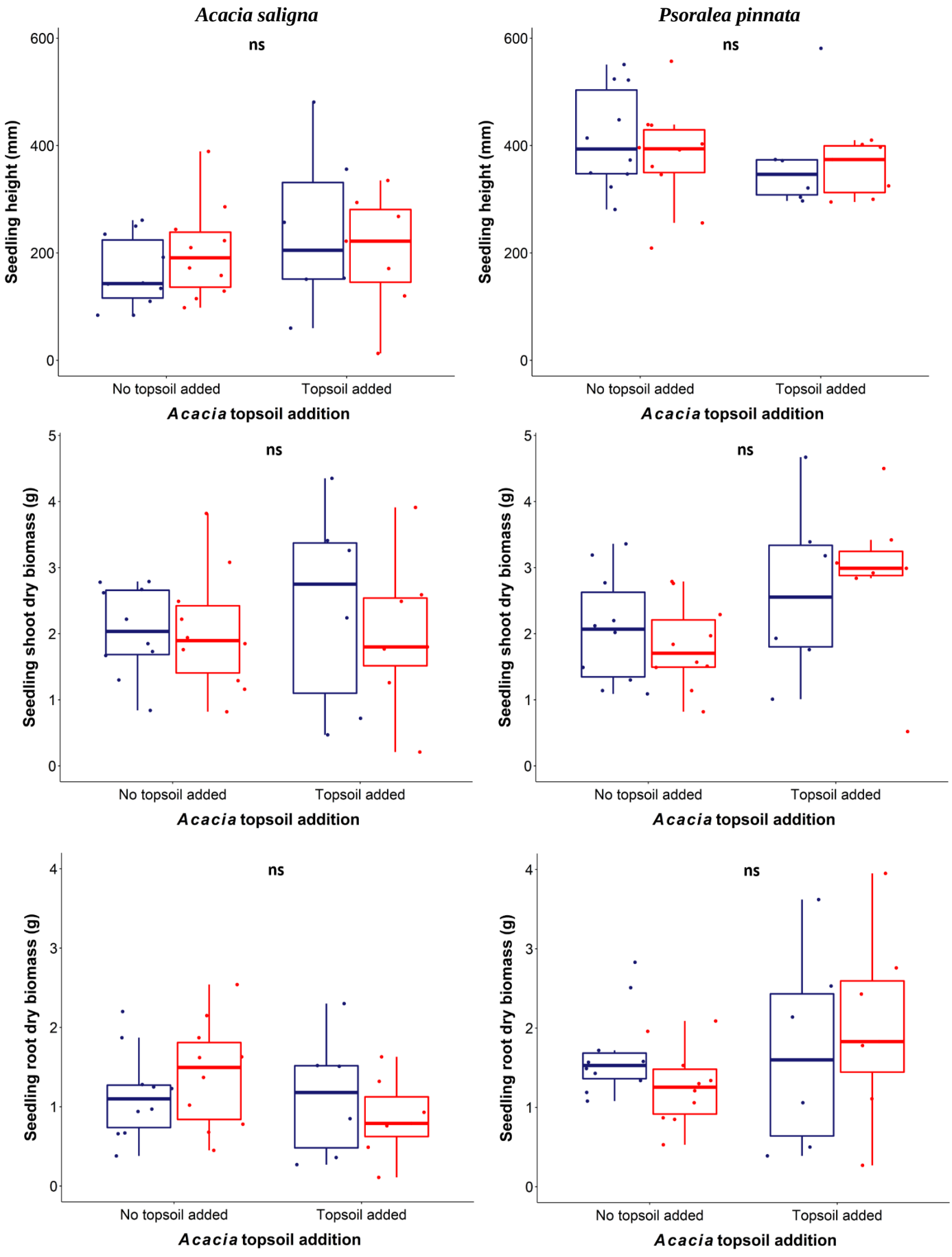


***Figure 3.2**: Relative Competition Index (RCI) values for growth performance (seedling height, seedling shoot dry biomass and seedling root dry biomass) and BNF (number of nodules and $\delta^{15}N$) measurements for each species (*Acacia saligna*; Psoralea pinnata) by inoculum treatment (red – Australian inoculum added; blue – no inoculum added) combination. The broken*

*horizontal line indicates where RCI=0, at which point seedlings in both competition treatments performed equally (no competitive interaction). RCI>0 indicates where seedlings grown alone outperformed seedlings grown in mixture (competition), and RCI<0 indicates where seedlings grown in mixture outperformed seedlings grown alone (facilitation). The * indicates which RCI values for each host species by inoculum treatment combination is significantly different to zero.*

***Table 3.2**: Results of ANOVAs of fixed effects for generalized linear mixed models comparing the Relative Competition Indices (RCI) for the different growth performance and BNF measurements between the species (*Acacia saligna *and* Psoralea pinnata*) and inoculum addition treatment combinations. Results for type I sum of squares are given for RCI values of seedling height, seedling shoot biomass and $\delta^{15}N$, while results of type III sum of squares are given for seedling root biomass and nodule number.*

	Type I sum of squares	numDf	denDf	F-value	p-value
Seedling shoot length	Intercept	1	18	47.8868	<0.0001
	Inoculum addition	1	18	0.9451	ns
	Species	1	13	27.4032	0.0002
	Inoculum addition: Species	1	13	0.4187	ns
Seedling shoot biomass	Intercept	1	18	110.25	<0.0001
	Inoculum addition	1	18	1.5736	ns
	Species	1	13	0.00713	ns
	Inoculum addition: Species	1	13	1.2088	ns
$\delta^{15}N$	Intercept	1	18	3.2827	ns
	Inoculum addition	1	18	4.6785	0.0442
	Species	1	13	1.8181	ns
	Inoculum addition: Species	1	13	0.0787	ns
	Type III sum of squares	**χ^2**	**df**	**p-value**	
Seedling root biomass	Intercept	15.282	1	<0.0001	
	Inoculum addition	0.0492	1	ns	
	Species	1.3035	1	ns	
	Inoculum addition: Species	13.5059	1	0.0002	
Nodule number	Intercept	12.6584	1	0.0004	
	Inoculum addition	0.0473	1	ns	
	Species	0.9657	1	ns	
	Inoculum addition: Species	4.5046	1	0.0338	



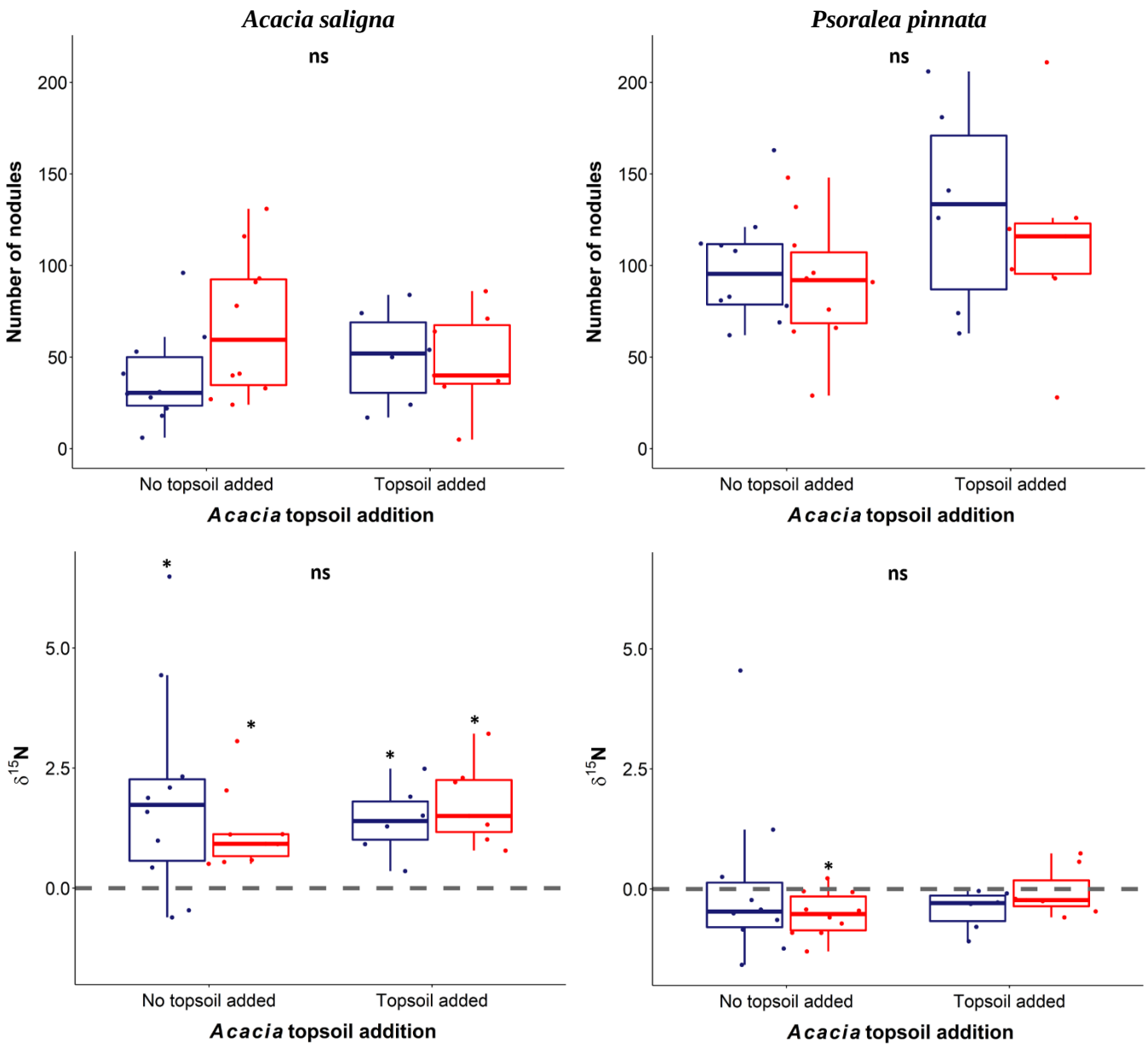


***Figure 3.3**: Growth performance (seedling height, seedling shoot dry biomass and seedling root dry biomass) and BNF (number of nodules and $\delta^{15}N$) measurements for* Acacia saligna *(left) and* Psoralea pinnata *(right) for each* Acacia*-topsoil addition by inoculum addition (red – Australian inoculum added; blue – no inoculum added) treatment combination. The broken horizontal line in the $\delta^{15}N$ graphs indicate where $\delta^{15}N = 0$. The * indicates which $\delta^{15}N$ values for each topsoil by inoculum treatment combination is significantly different to zero.*

***Table 3.3**: Results of ANOVAs of fixed effects for linear mixed models comparing the different growth performance and BNF measurements between different* Acacia*-topsoil addition and inoculum addition treatment combinations for* Acacia saligna *and* Psoralea pinnata.

		Acacia saligna				***Psoralea pinnata***			
		Num Df	Den Df	F-value	p-value	num Df	den Df	F-value	p-value
Seedling shoot length	Intercept	1	28	5.557	0.0256	1	28	95.6794	<0.0001
	Inoculum addition	1	28	0.0072	ns	1	28	1.3493	ns
	Topsoil addition	1	28	4.2912	0.0476	1	28	1.0852	ns
	Inoculum addition: Topsoil addition	1	28	1.2254	ns	1	28	0.0015	ns
Seedling shoot biomass	Intercept	1	28	2.8545	ns	1	28	18.4356	0.0002
	Inoculum addition	1	28	0.08308	ns	1	28	0.1833	ns
	Topsoil addition	1	28	3.9838	ns	1	28	6.2117	0.0189
	Inoculum addition: Topsoil addition	1	28	0.2747	ns	1	28	0.1241	ns
Seedling root biomass	Intercept	1	28	3.3934	ns	1	28	118.188	<0.0001
	Inoculum addition	1	28	0.0062	ns	1	28	0.1238	ns
	Topsoil addition	1	28	0.154	ns	1	28	1.7378	ns
	Inoculum addition: Topsoil addition	1	28	1.5747	ns	1	28	1.3378	ns
Number of nodules	Intercept	1	28	15.3807	0.0005	1	28	45.7384	<0.0001
	Inoculum addition	1	28	2.106	ns	1	28	0.8815	ns
	Topsoil addition	1	28	0.0014	ns	1	28	3.2656	ns
	Inoculum addition: Topsoil addition	1	28	7.9793	ns	1	28	0.3075	ns
$\delta^{15}N$	Intercept	1	28	16.2115	0.0004	1	28	0.0702	ns
	Inoculum addition	1	28	0.3447	ns	1	28	0.1784	ns
	Topsoil addition	1	28	0.0086	ns	1	28	0.0037	ns
	Inoculum addition: Topsoil addition	1	28	1.2122	ns	1	28	2.2298	ns

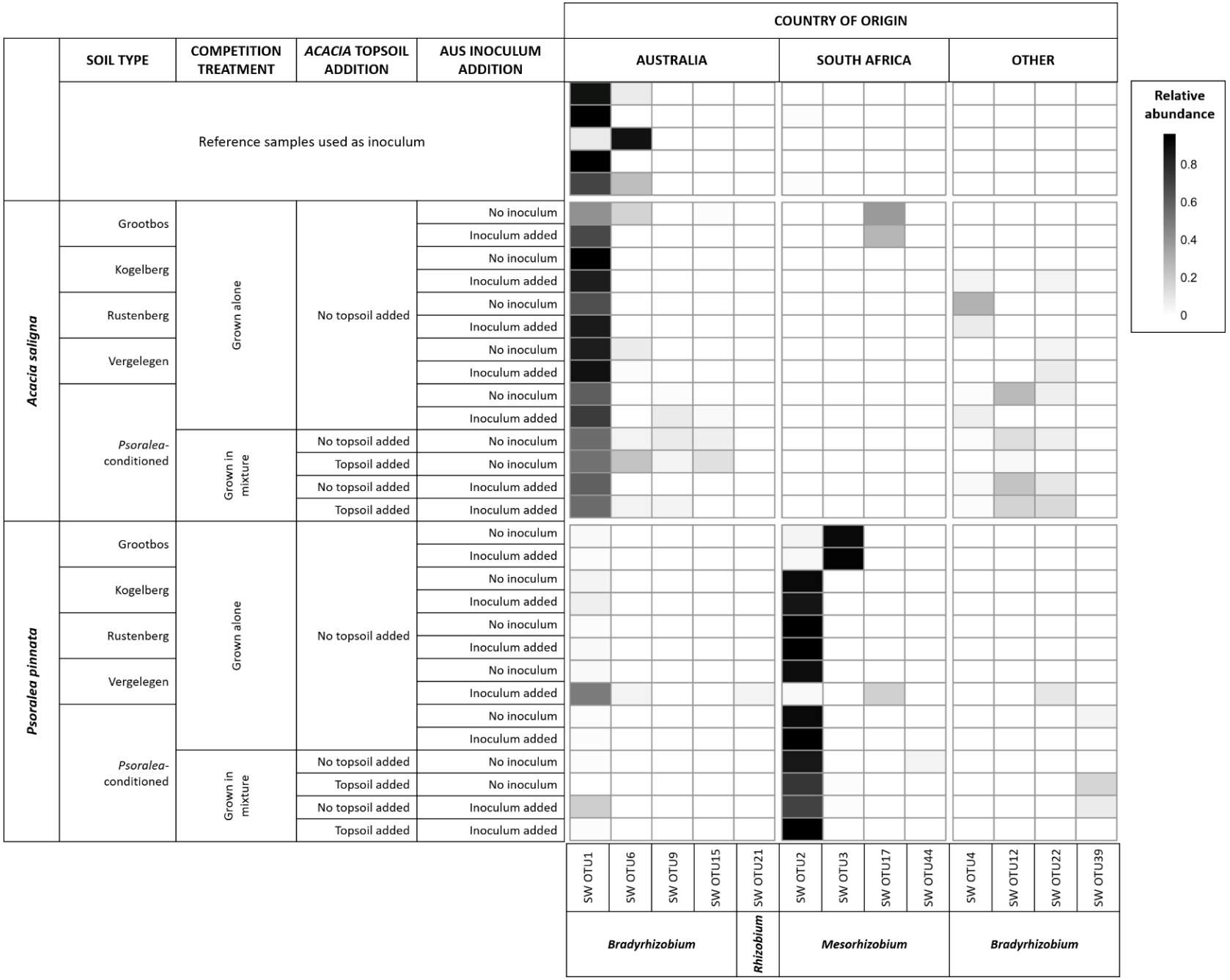


***Figure 3.4**: Heatmap based on the relative abundances of the rhizobial OTUs identified in this study (Chapter 2 and 3). Darker shades represent higher relative abundances. OTUs are arranged according to country of origin based on blast results and phylogenetic analyses (also see Fig. 2.5). Y-axis labels show experimental treatment combinations including soil type, competition treatment,* Acacia*-topsoil addition and Australian inoculum addition. OTU labels and genus identity based on blast results are shown on the bottom x-axis.*

Supplementary Materials

***Table S3.1**: Co-ordinates of sites for soil and* Acacia *topsoil collections*

Soil	Site	Co-ordinates
Psoralea-conditioned	Prawn river lagoon	-34.40743, 19.32842
	Kogelberg Nature Reserve	-34.17037, 18.95083
	Vergelegen Wine Farm	-34.05532, 18.94745
Acacia-topsoil	Vrede Wines	-33.85025, 18.80618

***Table S3.2**: Results of factorial ANOVAs comparing the different relative growth performance and BNF measurements of* Acacia saligna *between the different inoculum addition and topsoil addition treatment combinations.*

		Df	Sum of squares	Mean squares	F-value	p-value
Relative seedling height	Inoculum addition	1	0.0058	0.0058	0.281	ns
	Topsoil addition	1	0.0153	0.0153	0.737	ns
	Inoculum addition: Topsoil addition	1	0.0209	0.0209	1.007	ns
	Residuals	29	0.6021	0.0208		
Relative seedling shoot biomass	Inoculum addition	1	0.0017	0.0017	0.036	ns
	Topsoil addition	1	0.0449	0.0449	0.947	ns
	Inoculum addition: Topsoil addition	1	0.0091	0.0091	0.191	ns
	Residuals	29	1.3763	0.0475		
Relative seedling root biomass	Inoculum addition	1	0.0063	0.0063	0.121	ns
	Topsoil addition	1	0.0318	0.0318	0.610	ns
	Inoculum addition: Topsoil addition	1	0.0865	0.0865	1.661	ns
	Residuals	29	1.5111	0.0521		
Relative Number of nodules	Inoculum addition	1	0.0661	0.0661	1.770	ns
	Topsoil addition	1	0.0127	0.0127	0.341	ns
	Inoculum addition: Topsoil addition	1	0.0347	0.0347	0.930	ns
	Residuals	29	1.0821	0.0373		

Relative $\delta^{15}N$	Inoculum addition	1	0	<0,0001	0	ns
	Topsoil addition	1	0.0015	0.0015	0.057	ns
	Inoculum addition: Topsoil addition	1	0.005	0.005	0.184	ns
	Residuals	29	0.7853	0.0271		

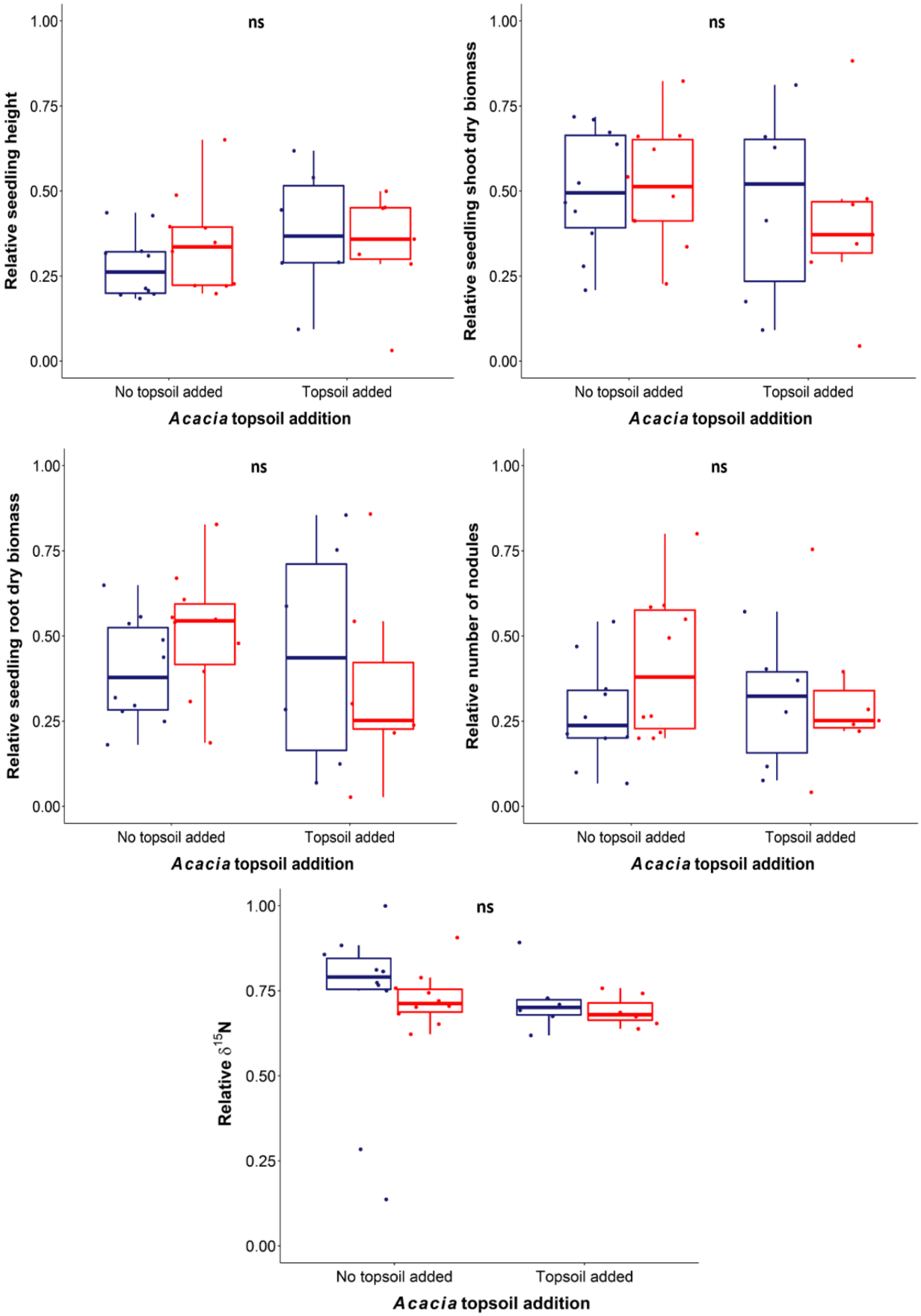


***Figure S3.1**: Relative growth performance and BNF measures of* Acacia saligna *for each topsoil addition by inoculum addition (red – Australian inoculum added; blue – no inoculum*

added) treatment combination. Higher values indicate dominance by Acacia saligna *while lower values indicate dominance by* Psoralea pinnata.

Chapter Four

Concluding remarks

Mutualisms are often vital for plants to complete their life cycles and their disruption during species introductions is considered a major barrier to the establishment success of non-native species (Parker, 2001; Richardson, Allsopp, *et al.*, 2000). The absence of effective rhizobial partners has been found to limit the success of non-native legumes (Gehlot *et al.*, 2013; Parker, 2001), yet, legumes are over-represented in the world's invasive flora (Pyšek *et al.*, 2017). This is because these legumes reassemble effective rhizobial mutualisms in their new ranges (Europe – Rodríguez-Echeverría *et al.*, 2009; Asia – Le Roux *et al.*, 2009; Southern Africa – Ndlovu *et al.*, 2013; Le Roux *et al.*, 2016; the Americas – Aronson *et al.*, 1992; New Zealand - Weir *et al.*, 2004), either by forming associations with resident mutualists (i.e. novel associations) or by co-invading with mutualists from their native ranges (i.e. familiar associations). Australian acacias have been shown to make use of both these mutualist reassembly pathways (Crisóstomo *et al.*, 2013; Ndlovu *et al.*, 2013; Rodríguez-Echeverría, 2010; Warrington *et al.*, 2019). The association with rhizobia has been suggested as a driving force behind Australian acacia establishment success as well as the accrual of impacts on the native communities in low nutrient environments (Le Maitre *et al.*, 2011; Morris *et al.*, 2011) by allowing efficient nutrient acquisition to support their increased growth rates which, in turn, drive positive feedback mechanisms that alter soil (a)biotic conditions (Kamutando *et al.*, 2019; Slabbert *et al.*, 2014; Yelenik *et al.*, 2004)

This thesis aimed to determine whether familiar rhizobial associations facilitated *A. saligna* establishment in different pristine soils from within the CCR. Furthermore, I also investigated the relative contribution of familiar rhizobial associations and positive feedbacks driven by leaf litter input (i.e. *Acacia*-conditioned topsoils) to the successful establishment of *A. saligna* under a competition scenario with the native legume, *Psoralea pinnata*. All my observational (i.e. DNA barcoding) and experimental (i.e, glasshouse experiments) data indicated that *A. saligna* associated with its preferred Australian *Bradyrhizobium* strains in all treatments. I also found significant differences in growth performances for *A. saligna* between the different soil types. These were matched by similar differences in growth for *P .pinnata*. Additionally, I found evidence that *A. saligna* and *P. pinnata* compete for resources, however,

A. saligna was not the dominant competitor. However, the competitive ability of *A. saligna* is nonetheless impressive considering that seedlings were grown in *Psoralea*-conditioned soils which should have afforded *P. pinnata* a competitive advantage.

The consistent association of *A. saligna* with Australian bradyrhizobia suggests that these exotic rhizobia are already present and widespread in pristine CCR soils. Together with the known history of cointroductions of acacias and their rhizobia within this region (Ndlovu *et al.*, 2013; Warrington *et al.*, 2019), this suggests that ongoing acacia spread and/or new introductions of Australian acacias may not be limited in the CCR by symbiont availability (Parker, 2001; Wandrag *et al.*, 2020). Indeed, cointroductions of Australian *Bradyrhizobium* have also been documented in Portugal (Crisóstomo *et al.*, 2013; Rodríguez-Echeverría, 2010) and New Zealand (Warrington *et al.*, 2019). Therefore, future studies should investigate the prevalence of *Acacia-Bradyrhizobium* cointroductions in invaded ranges in order to elucidate which regions may be more susceptible to acacia spread.

Since rhizobial availability is no longer limiting, one of the few remaining barriers to acacia establishment within the CCR would be the suitability of other soil (a)biotic conditions to acacia survival and growth, as well as the survival of the Australian bradyrhizobia. In fact, my results demonstrate that acacias experienced significant differences in growth performances between the different CCR soil types, regardless of the presence of Australian bradyrhizobia. While the survival of different rhizobial strains depend on soil conditions (Dludlu *et al.*, 2018a), such as pH, *Bradyrhizobium* (Rodríguez-Echeverría *et al.*, 2003) and *Mesorhizobium* (Dludlu *et al.*, 2018a) (the preferred symbiont of *P. pinnata*; Kanu and Dakora, 2012; Lemaire *et al.*, 2015) have broad pH tolerances and cosmopolitan distributions. Furthermore, there was successful nodulation for both species in all soils. Therefore, it is likely that instances where growth performances differed between these two species were driven by either increased pathogen loads (Thrall *et al.*, 2007) or differences in soil edaphic characteristics (*A. saligna* – Bar (Kutiel) *et al.*, 2004; *P. pinnata* – Bello *et al.*, 2017). Within the CCR, legume assemblages have been found to be driven by distinct edaphic characteristics (Dludlu *et al.*, 2018b), therefore, these are also likely to determine *A. saligna* establishment success. In fact, acacia nodulation and establishment success was also found to be largely determined by soil type when inoculuated with different soils from California (Klock *et al.*, 2016). These authors also

attributed differences in acacia performance to differences in soil characteristics and pathogen loads.

Interestingly, differences in growth performances between the different soil types were not dependent upon whether *A. saligna* was utilizing atmospheric nitrogen through BNF. In fact, in some instances, such as when grown in *Psoralea*-conditioned soils, *A. saligna* had the highest performances and yet these seedlings had high δ^{15}N values indicating less reliance on BNF than on soil nutrient for nitrogen assimilation. This is further highlighted in the competition experiment where *A. saligna* seedlings were grown in mixture with *P. pinnata* and were still assimilating soil nitrogen rather than making use of BNF. *Psoralea pinnata* is known to increase the soil nutrient contents in areas where its populations are well-established (Stirton *et al.*, 2015). Furthermore, *A. saligna* is efficient at assimilating available nutrients, having evolved in one of the most nutrient-poor environments in the world (Young & Young, 2001), and is also efficient at nutrient acquisition at high nutrient levels (Witkowski, 1991). This, together with the fact that BNF is more energetically expensive than soil nitrogen assimilation (Graham, 1992), would explain the lack of BNF in *Psoralea*-conditioned soils for *A. saligna* and may afford it a competitive advantage against *P .pinnata*, which was utilizing BNF, in the long-run. This is also a testament to the benefits derived from soil nutrient enrichment by acacia-driven positive feedbacks. While I found limited support for the facilitation of acacia topsoil on *A. saligna* performance, or the hindrance of *P. pinnata* performance through allelopathy, previous research has found acacia seedling establishment to be more successful when grown in soils collected from underneath acacia plants than from uninvaded sites, regardless of rhizobial inoculum application (Le Roux *et al.*, 2018). These changes, leading to positive feedbacks, accumulate over long time periods (Yelenik *et al.*, 2004). Therefore, it is likely that the lack of a facilitatory effect of *Acacia*-topsoil addition in my experiments was due to the short duration of the study. Another aspect which should be taken into consideration is how this increased soil nitrogen acquisition ability of *A. saligna* may provide a further advantage in exploiting the nitrogen flush that occurs post-fire in CCR communities. Germination of Australian acacia seeds are known to be activated by fire and, together with high seed loads and fast growth rates, the increased soil nitrogen acquisition ability of *A. saligna* may prove detrimental to other native CCR reseeders/resprouters reliant on the soil nitrogen deposition that accompanies fire (Witkowski, 1991).

In terms of the impacts of exotic bradyrhizobia and *A. saligna* competition on *P. pinnata* performances, it appeared that this native CCR legume was largely unperturbed by either of these factors. My results showed that this is likely driven by the ability of *P. pinnata* to successfully sanction associations with exotic *Bradyrhizobium* strains while preferentially selecting for *Mesorhizobium*. While associations with *Paraburkholderia* and *Rhizibium* have been recorded for this species before, their preferred symbiont is *Mesorhizobium* (Kanu and Dakora, 2012; Lemaire *et al.*, 2015). Additionally, *P. pinnata* was not out-competed by *A. saligna* and was not negatively impacted by acacia topsoil. This is in line with observations of *P. pinnata* occurring alongside invasive acacia stands as well as the regeneration of this species through passive restoration efforts after acacia stands have been cleared (Reinecke *et al.*, 2008). Furthermore, *P. pinnata* have similar functional traits to *A. saligna*, such as rapid post-fire germination and increase growth rates (Stirton *et al.*, 2015). While this highlights the benefits of *P. pinnata* for restoration projects within the CCR, altogether, this also raises a red flag in terms of this species' potential as an invader, particularly in areas where they have already naturalized, such as Australia (Stirton *et al.*, 2015). However, this competitive integrity in the face of an Australian acacia appears to be an exception rather than the rule. Le Roux *et al.* (2016) found that other genera of CCR legumes were not able to successfully sanction exotic bradyrhizobia and their nodules harboured compositionally different rhizobial communities when compared between acacia-invaded and uninvaded sites. Additionally, non-leguminous CCR shrubs are often outcompeted by *A. saligna* due to their slower growth rates and later post-fire seed germination strategies (Witkowski, 1991). Similar biodiversity declines due to acacia superior competitive abilities have been found in other Mediterrainean areas, such as the coastlines of Portugal (Marchante, Marchante & Freitas, 2003) and Italy (Del Vecchio, Acosta & Stanisci, 2013). Therefore, future studies should consider investigating the sanctioning abilities of *P. pinnata* in greater detail as well as its ability to establish in *Acacia*-conditioned soils as this species has the potential to be utilized in active restoration projects post-acacia clearing with potential above- and belowground positive impacts.

Despite their complexity and context-dependency, understanding the role of biotic interactions, such as mutualisms, in invasion dynamics is much needed (Novoa *et al.*, 2020). For Austrailian acacias, strong patterns have recently emerged (Keet *et al.*, 2017; Lorenzo *et al.*, 2017; Le Roux *et al.*, 2018; Wandrag *et al.*, 2020; Yannelli *et al.*, 2020), and my research contributes to these. Adding to a growing body of research, my study confirms that rhizobium

mutualisms are not limiting acacia invasions as cointroductions of their preferred bradyrhizobia ensures availability of effective rhizobial partners in many non-native ranges (Weir *et al.*, 2004; Rodríguez-Echeverría, 2010; Crisóstomo, Rodríguez-Echeverría and Freitas, 2013; Ndlovu *et al.*, 2013; Birnbaum *et al.*, 2016; Warrington *et al.*, 2019; Wandrag *et al.*, 2020). My research suggests that the time is ripe to consider the context within which mutualist associations occur and how different soil conditions may alter the supposed symbiotic benefits accrued by non-native acacias. Future studies should investigate the causal mechanisms driving the differences between different soil types I reported here and how these mutualistic benefits may be altered under different soil (a)biotic conditions.

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